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Analysis of sophorolipids from *Candida bombicola*

by

Albert Lumanlan Ussher

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

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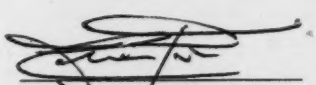

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Analysis of sophorolipids from *Candida bombicola* by Albert Lumanlan Ussher in partial fulfillment of the requirements for the degree of Master of Science in Microbiology and Biotechnology.

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Abstract

Candida bombicola is capable of producing sophorolipids when grown in medium that includes nitrogen, lipidic and glucidic carbon sources. Sophorolipids have been commercially applied as biosurfactants. Purity of sophorolipids was analyzed in this study, in which *C. bombicola* was cultivated under batch conditions. The growth medium consisted of yeast extract, sunflower oil and glucose. Fermentation of 20 days was necessary to produce sophorolipids of high purity and yield (45 g/L). Yield coefficients for sophorolipids and biomass were determined to be 0.25 and 0.033, respectively. Prior to 20 days, the sophorolipids contained impurities observed by thin layer chromatography. Gas chromatography separated these into nine peaks, and mass spectrometry was used to identify oleic acid, and tentatively identify stearic and linoleic acids. The main repercussion of this result is the cost of long fermentation times. However, the alternate of shorter times would incur further costs in removing these impurities.

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List of Abbreviations

Ac	acetate
ATCC	American Type Culture Collection
C. bombicola	*Candida bombicola*
cm	centimeter
CMC	critical micelle concentration
C_{max}	maximum concentration of fatty acid during fermentation (g/L)
C_0	concentration of fatty acid at time zero (g/L)
C_t	concentration of fatty acid during sampling time of fermentation
C_{xm}	maximum biomass yield
d	day
DCM	dry cell mass
FAME	fatty acid methyl ester
g/L	gram per liter
GC	gas chromatography
g_P/g_S	gram sophorolipids per gram substrates consumed
h	hour
H	hydrogen atom
HCl	hydrochloric acid
HPLC	High performance liquid chromatography
IEC	International Equipment Company

kPa	kiloPascal
LC	liquid chromatography
M	molar concentration
m/m	mass to mass ratio
m/v	mass to volume ratio
m/z	mass to charge ratio, used in mass spectrometry
min	minute
mg/L	milligram per liter
mL	milliliter
mm	millimeter
mN/m	milli-Newtons per meter, measurement unit of interfacial and surface tensions
MS	mass spectrometry or mass spectrum
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
nm	nanometer
NMR	nuclear magnetic resonance
°C	degree Celsius
OD	optical density of cell culture
P	product, used to represent sophorolipid yield
PTFE	polytetrafluoroethylene, used in stopcock of separatory funnel
R	generic term for H or carbon side chain
R_f	rear to front ratio, used in TLC
rpm	revolutions per minute

s	second
S	substrates consumed, glucose and oil
Sl	sophorolipids
TLC	thin layer chromatography
UK	United Kingdom of Great Britain
US$/kg	United States dollars per kilogram
UV	ultraviolet light
v/v	volume to volume ratio
v/v/v	volume to volume to volume ratio
v/v/min	volume of air to volume of liquid to minute ratio, unit, used to represent aeration of stir tank fermenters
X	biomass of *C. bombicola*
y	year
$Y_{CO2/S}$	yield coefficient of carbon dioxide with respect to substrates consumed
YM	yeast-malt extract
$Y_{P/S}$	yield coefficient of sophorolipids to substrates consumed
$Y_{P/X}$	yield coefficient of sophorolipid to biomass produced
$Y_{X/S}$	yield coefficient of biomass to substrates consumed
12:0	lauric acid or dodecanoic acid
14:0	myristic acid or tetradecanoic acid
16:0	palmitic acid or hexadecanoic acid
16:1	palmitoleic acid or 9-hexadecenoic acid
18:0	stearic acid or octadecanoic acid

18:1	oleic acid or 9-octadecenoic acid
18:2	linoleic acid or 9,12-octadecadienoic acid
18:3	linolenic acid or 9,12,15-octadecatrienoic acid
20:0	arachidic acid or docosanoic acid
20:1	gadoleic acid or 11-eicosenoic acid
20:2	gadolenic acid or 11,14-eicosadienoic acid
22:0	behenic acid or docosanoic acid
22:1	cetoleic acid or 13-docosenoic acid
24:0	lignoceric acid or tetracosanoic acid
24:1	nervonic acid or 15-tetracosanoic acid
%	percent
μ	specific growth rate
μL	microliter
μm	micrometer
μ_p	specific sophorolipid production rate

1.0 Background

1.1 Introduction

J. F. T. Spencer (Prairie Regional Laboratory, Saskatchewan, Canada) first isolated *C. bombicola* in 1954 from sow thistle petals (Spencer *et al.*, 1979). This organism was also isolated from several bumblebee nests in Western Canada. The name "*bombicola*" was derived from the word bumblebee. *C. bombicola* was classified in the family of Yeast Imperfecti. It was soon discovered that these organisms produced extracellular oil that was denser than water when cultivated in liquid broth medium. These molecules were characterized as sophorolipids (Gorin *et al.*, 1961). Sophorolipids are one type of biosurfactants.

1.1.1 Definition of biosurfactants

Biosurfactants are biologically produced surfactants, and they are produced mainly by aerobically growing microorganisms in aqueous media from a carbon source feed stock such as mixtures of carbohydrates, hydrocarbons, oils, and fats (Bognolo, 1999). By definition, surfactants are amphiphilic molecules composed of hydrophilic and hydrophobic domains (Banat *et al.*, 2000). The lipophilic moiety of biosurfactants can be a protein or peptide with a high proportion of hydrophobic side chains, but is usually the hydrocarbon chain of a fatty acid with 10 to 18 carbon atoms. On the other hand, the hydrophilic group can be an amino acid, ionic peptide, ester, hydroxyl, phosphate, carboxylate, mono-, di-, or polysaccharides (Bognolo, 1999; Desai and Banat, 1997).

Surfactants are surface-active agents (Ron and Rosenberg, 2001) and as such are capable of reducing the surface and interfacial tension of the interfaces of liquids, solids and gases (Banat *et al.*, 2000). Interfacial tension is the force that keeps these surfaces apart or causes them to coalesce. There is no interfacial tension between surfaces of the same liquid, solid or gas. Surface tension is a special case of interfacial tension and describes the contact between solids or liquids with gases (Young and Coons, 1945). For example vegetable oil and water are unable to mix, because of high interfacial tension between the two liquids. In comparison, chloroform and methanol are miscible due near zero interfacial tension. The water-air contact is the classic example of surface tension. An ideal surfactant would reduce the interfacial and surface tension between these cases and allow the mixing of immiscible solids, liquids and gases. This ability to reduce interfacial tension is the major driving force in the application of surfactants. The creation of novel surfactants is attributed to the diverse combinations of immiscible solid, liquid and gases.

Biosurfactants are classified according to their chemical composition and their microbial origin. There are several major classes of biosurfactants including, but not limited to glycolipids, lipopeptides and phospholipids. Glycolipids are the most studied biosurfactants, and are composed of a carbohydrate in combination with long chain aliphatic acids or hydroxylated aliphatic acids (Desai and Banat, 1997). Glycolipids are typically involved in the uptake of low polarity hydrocarbons such as alkanes by microorganisms (Bognolo, 1999). The best-known glycolipids (in decreasing order) are rhamnolipids, trehalolipids and sophorolipids. Rhamnolipids and trehalolipids are produced by *Pseudomonas* and *Mycobacterium* species respectively. In comparison,

many yeasts in the *Candida* genus produce sophorolipids, including *C. bombicola*, *C. apicola*, *C. bogoriensis*, and *C. petrophilum*. *C. bombicola* is the most studied of all the sophorolipid producing yeasts (Desai and Banat, 1997) followed by *C. apicola* (Hommel *et al.*, 1994a, 1994b, 1994c, Hommel and Huse, 1993; Hommel *et al.*, 1987). The reader is encouraged to seek the numerous reviews on biosurfactants for more detail (Ron and Rosenberg, 2001; Banat *et al.*, 2000; Bognolo, 1999; Makkar and Cameotra, 1999; Rosenberg and Ron, 1999; Desai and Banat, 1997; Lin, 1996; Desai and Desai, 1993; Hommel and Ratledge, 1993; Lang and Wagner, 1993; Fiechter, 1992; Georgiou *et al.*, 1992; Syldatk and Wagner, 1987; Cooper and Zajic, 1980; Stodola *et al.*, 1967).

1.1.2 Market analysis of biosurfactants

The worldwide market for surfactants has been estimated at 9.4 billion US$/y (Desai and Banat, 1997). For example, the US produces over 250 x 10^6 kg of surfactants annually. The market value of these surfactants was estimated at over 1.8 billion US$ (United Soybean Board, 2002; Colin A. Houston and Associates, 1998). A break down of this market showed that anionic, nonionic and cationic surfactants accounted for 29 %, 26 % and 11 % respectively (Colin A. Houston and Associates, 1998). Moreover, surfactants utilized in household laundry detergents and industrial applications accounted for 54 % and 32 % respectively (Banat *et al.*, 2000). The overwhelming majority of these surfactants were chemically synthesized and mainly derived from petroleum.

There are several reasons why biosurfactants should capture a portion of this market. Advantages of biosurfactants over chemical surfactants are biodegradability, lower

toxicity and biocompatibility, and activity at a wide range of pH, temperature and salinity (Desai and Banat, 1997). The majority of biosurfactants produced are anionic and nonionic molecules, and both molecules combined make up over 50 % of the US surfactant market. An increase in environmental awareness followed by legislation for environmentally friendly products has opened other markets for biosurfactants (Banat et al., 2000; Bognolo, 1999; Rosenberg and Ron, 1999). The cost of biosurfactants has been the most important deterrent to complete c ommercialization. Chemical surfactants a re typically sold for 3.3 US$/kg to 6.6 US$/kg. Some specialty surfactants run from around 2 US$/kg to over 220 US$/kg (Colin A. Houston and Associates, 1998). One estimate placed the cost of biosurfactants at 3 US$/kg to 20 US$/kg (Bognolo, 1999). More specifically, sophorolipids have been estimated at 2 US$/kg, making these biosurfactants attractive for commercial application (Deshpande and Daniels, 1995).

1.1.3 Sophorolipid applications

There are numerous and diverse "potential" applications for biosurfactants including hydrocarbon bioremediation, oil storage tank cleaning, enhanced oil recovery, therapeutic agents, agriculture adjuncts, personal care and hygiene, and food additives (Banat *et al.*, 2000). The focus of this section is to reveal "actual" applications of sophorolipids through examples of existing patents. The majority of patents on the applications of sophorolipids are in cosmetics and detergents. Sophorolipids have been used in cosmetics such as lotions and skin creams (Borzieux, 2000; Thorel *et al.*, 1998; Hayes, 1991). More specifically, sophorolipids have been used in cosmetic formulations of naturally occurring surfactants (Thorel *et al.*, 1998). In these patents, claims have been made that

when applied on the skin, sophorolipids can moisturize, condition, and protect dry skin (Borzieux, 2000; Hayes, 1991). Topically applied therapeutics included sophorolipids as the active ingredient for the treatment of the skin (Maingault, 1999). These have been shown to have uses in anti-inflammation, and the inhibition of radical formation and elastase activity (Hillion *et al.*, 1998).

Detergent formulations have also included the use of sophorolipids. These have been found to be effective in liquid detergent composition for washing dishes or textiles by hand (Bergeron, 2001). Detergents used in washing machines have also included sophorolipids in their formulations (Clark *et al.*, 2001; Guerin *et al.*, 2001; Crawford and Sanderson, 1997). Sophorolipids combined with sodium lauryl sulfate have been shown to have effective germicidal properties. More specifically, 100 % kills of *Escherichia coli*, *Salmonella* and *Shigella* within 30 s have been shown, after application to the surface (Pierce and Heilman, 2001).

There are a few cases of industrial application of sophorolipids. Sophorolipids have been included in surfactant formulations in soil bioremediations (Ducreux *et al.*, 1997). Compositions of bio-emulsifiers to facilitate the transportation of hydrocarbons in pipes have included the use of sophorolipids (Hayes *et al.*, 2000). These have been shown to inhibit cracking of metallic pipes used to transport petroleum and crude oils under sulfide stress (Anderson *et al.*, 1989). The textile and pad dyeing industry have used a formulation that included sophorolipids that replaced urea. Urea tended to accumulate in the wastewater and was less environmentally friendly (Schumacher *et al.*, 1998). Lastly,

sophorolipids have been used as a source of sophorose as an alternative to chemical synthesis of this disaccharide (Ernst *et al.*, 1994).

1.2 Sophorolipids

1.2.1 Structure and nomenclature

The elucidation of the sophorolipid structure has been the initial stumbling block into the study of sophorolipids. Aspects of this problem include the structure of the sophorose molecule, types of fatty acid tails, the site of hydroxylation on the fatty acid tail, and the macrocyclic lactone. Authors have used various methods in analyzing the structure of sophorolipids. Past analyses of sophorolipid structures often involved results of predictable and specific chemical reactions (Gorin *et al.*, 1961, 1962; Tulloch *et al.*, 1962, 1968; Tulloch and Spencer, 1967, 1972; Heinz *et al.*, 1969, 1970, Spencer *et al.*, 1979). Present structural analyses make less use of these reactions and rely on ^{13}C and ^{1}H 1D and 2D NMR, HPLC, GC, and MS in the elucidation of sophorolipid structure (Asmer *et al.*, 1988; Davila *et al.*, 1992, 1995, 1997, Bisht *et al.*, 1999; Nunez *et al.*, 2001).

Gorin *et al.* (1961) introduced the nomenclature of sophorolipids. However, most authors have used the nomenclature introduced by Davila *et al.*, 1993. (Table 1.1) shows the traditional as well as common nomenclature of major sophorolipids. Figure 1.1 matches the nomenclature with the structure of the corresponding sophorolipid. The sophorose molecule of the sophorolipid is not affected by different glucidic substrate (Esders and Light, 1972; Gobbert *et al.*, 1984). Glucose is the preferred substrate for the biosynthesis

of the sophorose molecule, although *C. bombicola* can grow in various carbohydrates. Glucidic substrates that have been used in the production of sophorolipids include glucose, sucrose, fructose, and mannose (Klekner *et al.*, 1991; Zhou and Kosaric, 1993; Gobbert *et al.*, 1984). Oleic acid is also the preferred fatty acid for the incorporation into the lipidic backbone of the sophorolipids, although other fatty acids and hydrocarbons can be incorporated into the lipidic backbone. More specifically, higher concentrations of oleic and stearic acids in the vegetable oil favored the production of diacetylated lactone sophorolipids (Davila *et al.*, 1994). Lastly, the site of the majority of the glycosidic bonding tended to occur on the penultimate carbon of the fatty acid with minor bonding occurring to the ultimate carbon (Gorin *et al.*, 1961, 1962; Tulloch *et al.*, 1962, 1968; Tulloch and Spencer, 1967, 1972; Heinz *et al.*, 1969, 1970, Spencer *et al.*, 1979).

Table 1.1 Nomenclature of major sophorolipids. (Davila *et al.*, 1993). See Figure 1.1 for structures.

Common	Traditional	Link
Sophorose	2-O-β-D-glucopyranosyl-β-D-glucopyranose	Fig1
Diacetylated acidic sophorolipids or acid 6' 6"-diacetate	17-L- ((2'-O-β-D-glucopyranosyl)-oxy)-9-octadecenoic acid 6', 6" diacetate	Fig 1A, R'=R"=Ac
Acetylated acidic sophorolipids or acid 6'-monoacetate	17-L- ((2'-O-β-D-glucopyranosyl)-oxy)-9-octadecenoic acid 6' monoacetate	Fig 1A, R'=Ac, R"=H
Non-acetylated acidic sophorolipids or acid sophorolipids	17-L- ((2'-O-β-D-glucopyranosyl)-oxy)-9-octadecenoic acid	Fig 1A, R'=R"=H
Diacetylated lactones or 1', 4"-lactone 6', 6"-diacetate	17-L- ((2'-O-β-D-glucopyranosyl)-oxy)-9-octadecenoic acid 1', 4"-lactone 6', 6" diacetate	Fig 1B, R'=R"=Ac
6'-acetylated lactones or 1', 4"-lactone 6'-monoacetate	17-L- ((2'-O-β-D-glucopyranosyl)-oxy)-9-octadecenoic acid 1', 4"-lactone 6' monoacetate	Fig1B, R'=Ac, R"=H
6"-acetylated lactones or 1', 4"-lactone 6"-monoacetate	17-L- ((2'-O-β-D-glucopyranosyl)-oxy)-9-octadecenoic acid 1', 4"-lactone 6" monoacetate	Fig 1B, R'=H, R"=Ac
Non-acetylated lactones or 1', 4"-lactone	17-L- ((2'-O-β-D-glucopyranosyl)-oxy)-9-octadecenoic acid 1', 4"-lactone	Fig 1B, R'=R"=H

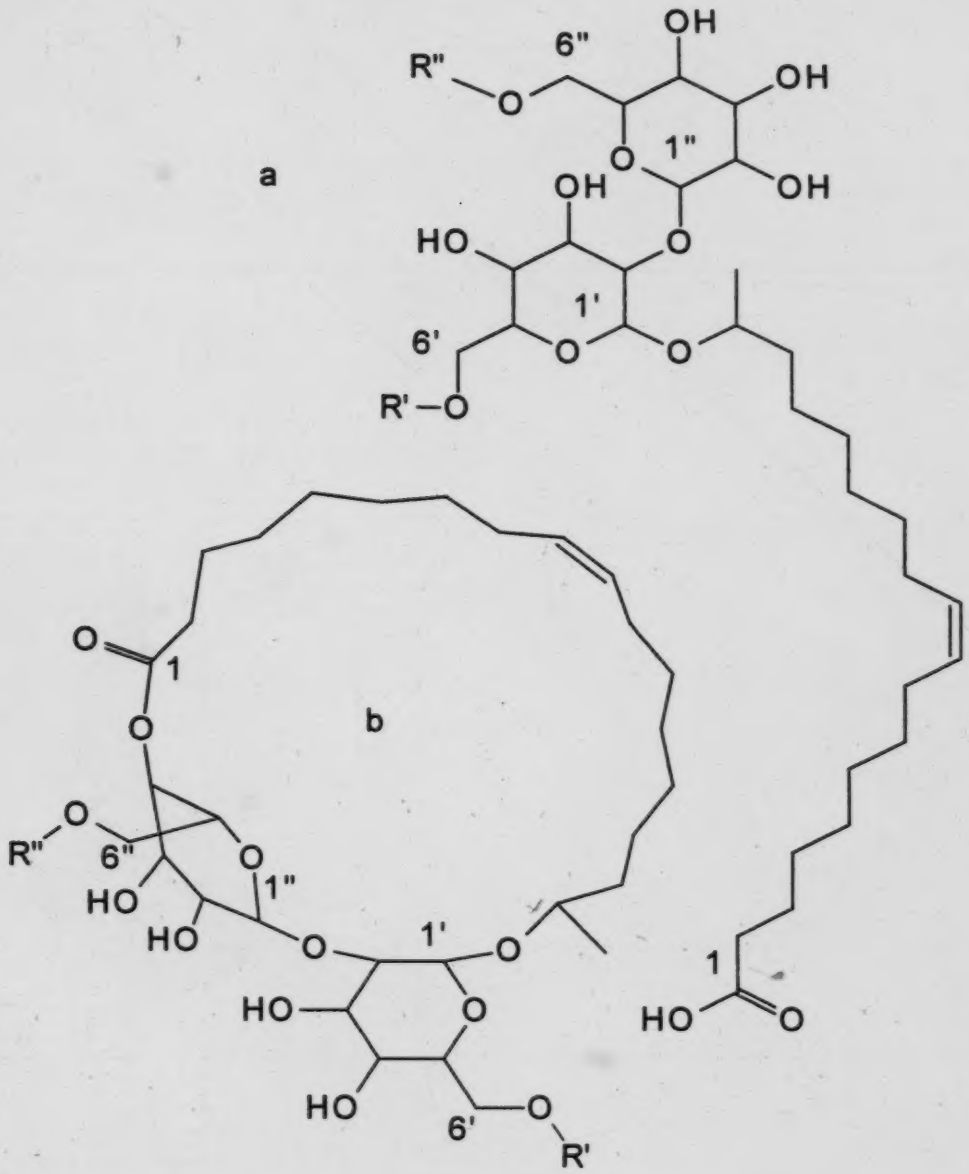

Figure 1.1 Structural classes of major (a) acid and (b) lactone sophorolipids. Note: R'= Ac or H; R" = Ac or H (Davila *et al.*, 1993). See Table 1.1 for nomenclature.

1.2.2 Sophorolipid composition

The literature data on the composition of sophorolipids produced by *C. bombicola* have been very sparse. The composition of sophorolipids is reported by the quantity of diacetylated or total lactones in the mixture, because lactones constitute the majority of sophorolipids produced in batch fermentations (Deshpande and Daniel, 1995; Klekner *et al.*, 1991; Zhou *et al.*, 1992; Zhou and Kosaric, 1995). In batch fermentations, the total lactones have been reported at 65 % of all sophorolipids extracted by ethyl acetate (Casas and Garcia-Ochoa, 1999). Moreover, there have been several reports on the diacetylated lactone composition of sophorolipids varying from 45 %, 65 %, 73 %, and 79 % of extracted sophorolipids (Deshpande and Daniels, 1995; Zhou *et al.*, 1992; Zhou and Kosaric, 1995; Klekner *et al.*, 1991).

The effect of fed-batch fermentation on the composition of sophorolipids is also not well studied. Asmer *et al.* (1988) and Davila *et al.* (1992, 1993, 1994, and 1997) analyzed the composition of all lactones in fed-batch reactions. The diacetylated lactones and total lactone composition were 62 % and 74 %, respectively when glucose and oleic acid were used. In comparison, 40 % and 59 % as diacetylated and total lactones were produced when oleic acid only was used (Asmer *et al.*, 1988). Davila *et al.* (1993) observed the diacetylated lactones and total lactones were 51 % and 72 %, respectively, when ethyl esters of rapeseed oil were used. The use of *C. bombicola* batch and fed-batch fermentations both led to over 50 % lactone production. However, less convergence was observed with the absolute concentration of lactones between batch and fed-batch. More

over, different lactone concentrations were observed within the batch and fed-batch methods of production. The effect of the type of fermentation on the composition of sophorolipids was inconclusive.

Other parameters that have been studied with respect to their effects on the composition of sophorolipids in batch fermentations are aeration, inoculum density, temperature, pH, fermentation time, and yeast extract concentration. Higher rate of aeration favor greater production of sophorolipids, however varying aeration does not have a significant effect on the composition of the mixture (Casas and Garcia-Ochoa, 1999). A denser inoculum produced a higher concentration of sophorolipids in less cultivation time than less dense inoculum. However, sophorolipid composition after 8 days of batch fermentation was similar between cases of low and high inoculum densities (Casas and Garcia-Ochoa, 1999). Although the quantity of sophorolipids are optimum at 25 °C and 3.5 pH, the temperature and pH have no significant effect on the composition of the sophorolipid mixture (Davila *et al.*, 1997).

The elapsed time of cultivation during batch reaction also has conflicting effects on the composition of sophorolipid mixtures. In a medium used by Zhou *et al.* (1992), overall lactone composition did not change during cultivation. In contrast, the medium used by Casas and Garcia-Ochoa (1999) showed that longer fermentation times favored the production of lactones over acidic sophorolipids. Further analysis of the two studies suggests that medium containing mineral salts had a more stable production of lactones,

whereas medium a medium lacking these salts required more time to produce lactone sophorolipids.

Conflicting trends in lactone production are observed under batch conditions when yeast extract concentration is varied between 0.1 % and 2.0 %. Several groups observed that increasing yeast extract concentration from 0.1 % to 2.0 %led to an increase in the proportion of lactones in the sophorolipids. More specifically, Klekner *et al*. (1991) noticed an increase from 30 % to 79 % diacetylated lactones when ye ast extract was increased in the production medium containing sunflower oil. The variation of glucidic substrate between glucose and sucrose did not have an effect on the previous trend. Zhou *et al.*, (1992) noticed a similar trend when glucose and safflower oil were used for the glucidic and lipidic carbon sources, respectively. In comparison, one group observed a different trend, such that the total lactone composition decreased from 65 % to 10 % when yeast e xtract concentration w as increased (Casas and Garcia-Ochoa, 1999). All authors used glucose and vegetable oil in the production medium. It is difficult to make direct comparisons between the different trends, considering only the total lactone composition was studied in Casas and Garcia-Ochoa (1999), while ignored by the other authors.

1.2.3 Novel sophorolipids

The main driving force for the synthesis of novel sophorolipids has been the design of better biosurfactants. A biosurfactant can be commercially successful if it can be economically competitive with a comparable chemical surfactant, or if it possesses novel

applications not found in commercially available chemical surfactants. Several authors have shown that a mixture of sophorolipids reduce the surface tension of water from 72 mN/m to 30 mN/m and interfacial tension between hexadecane and water to 1 mN/m (Cooper and Paddock, 1984; Rau *et al.*, 1996). Thus successfully modified sophorolipids would reduce the surface tension below 30 mN/m or the interfacial tension to 1 mN/m between water and other organic compounds.

In the literature, there are two general techniques for producing novel sophorolipids. One method of producing novel sophorolipids utilizes unique lipidic substrates such as primary alcohol, secondary alcohols and ketones (Brakemeier *et al.*, 1995; 1997; 1998). The second method uses the existing sophorolipids produced from a conventional lipidic substrates and enzymatic modification of the sophorolipid (Asmer *et al.*, 1988; de Koster *et al.*, 1995; Bisht *et al.*, 1999; Otto *et al.*, 1999; Rau *et al.*, 1999). Initial fermentation with alkanes and primary alcohols shorter than 16 carbons has been unsuccessful, because these compounds were metabolized by *C. bombicola* and the sophorolipids were synthesized *de novo* (Jones, 1968; Brakemeier *et al.*, 1995). Increasing the biomass concentration prior to addition of these compounds solved this problem. Optimum conditions were achieved when glucose and yeast extract concentrations were increased to 15 % and 0.4 % respectively. Secondly, stationary phase cultures were necessary for bioconversion (Brakemeier *et al.*, 1995).

The majority of novel sophorolipids produced from 1-dodecanol (88 %) and 2-tetradecanol (78 %) was found to consist of the hydroxyl of the alcohol directly

incorporated into the glycosidic bond of the sophorose molecule. In some cases the ultimate or penultimate carbon of the alcohol was hydroxylated then incorporated into the sophorose molecule. Both novel sophorolipids were able to decrease the surface tension of water from 72 mN/m to 30 to 33 mN/m, thus making them comparable to commercial surfactants such as octylglycoside and beta-dodecylmaltoside (Brakemeier *et al.*, 1995 and 1997). The 2-, 3- and 4-dodecanone were used as co-substrates for novel sophorolipid production. The authors found that these ketones were reduced to the corresponding alcohol and incorporated directly into the sophorose molecule. Although the sophorolipid yields for 2- and 3-dodecanone were orders higher than 4-dodecanone (Brakemeier *et al.*, 1998).

The existing sophorolipids produced from conventional substrates are enzymatically modified in the second method. Di-acetylated lactone sophorolipids were converted to the de-acetylated form in the presence of acetyl esterase (EC 3.1.1.6). Unfortunately the reaction time was greater than 10 d. The maximum yield obtained was 30 %. A greater yield was not obtained, because the lactone ring and the glycosidic bonds were also attacked by the esterase (Asmer *et al.*, 1988). De Koster *et al.* (1995) re-visited the idea of removing acetyl groups from di-acetylated sophorolipids. Cutinase from the fungus *Fusarium solani* was used. Regioselective removal of the acetyl group from the 6' carbon was observed without affecting the lactone bond (de Koster *et al.*, 1995). Lipases from *Candida rugosa* and *Mucor miehei* were also found to remove the acetyl group from the 6' position without attacking the lactone bond. In this situation the reaction was complete after 48 h and 95 % conversion was obtained. The mono-acetylated lactone sophorolipid

had a similar critical micelle concentration as the di-acetylated form (de Koster *et al.*, 1995; Otto *et al.*, 1999). In contrast, the surface tension was not as effectively reduced by the mono-acetylated lactone sophorolipid in comparison to the di-acetylated form (Otto *et al.*, 1999).

Another attempt at enzymatic modification used the glycosidase called hesperidinase (EC 3.2.1.4). Of all the glycosidases tested the hesperidinase was observed to be the most successful at breaking the ether bond between 2' and 1" carbons (Figure 1.1). This reaction led to the release of one glucose molecule. The resulting glucolipid had very similar surface and interfacial properties as the acidic sophorolipids. In contrast, the glucolipid was less soluble in water than the acidic glycolipid (Rau *et al.*, 1999).

Lastly, an attempt at enzyme modification involved the lipase from *Candida antarctica* called Novozym 435. This enzyme was chosen after comparison with other lipases. De-acetylated acidic sophorolipids were first esterified to increase their solubility in organic solvents. Novozym 435 acetylated methyl esters of de-acetylated acidic sophorolipids in both 6' and 6" carbon positions. The acetyl donor used in this reaction was vinyl acetate. In contrast when vinyl acetate was removed from the reaction mixture, a different lactone was formed. The lactone bond was at the 6" carbon instead of the typical 4" (Figure 1.1). This lactone was a synthetic analogue of the one produced by *C. bombicola*. The conversion of this reaction was greater than 70 % (Bisht *et al.*, 1999). In comparison, *C. bombicola* naturally produced 6' or 6" lactones at less than 1 % of the total sophorolipids produced (Davila *et al.*, 1993).

1.3 Production of sophorolipids

Production of sophorolipids had been accomplished using various fermentation processes that include batch, fed-batch, continuous, self-cycling, two-stage, and resting cell methods. A wide range of sophorolipid yields was obtained under batch and fed-batch schemes of production (Table 1.2). All yields reported have used ethyl acetate to extract sophorolipids from the culture broth. The batch process had been used most commonly, because of its simplicity in comparison to the other processes. The main objective of all fermentation schemes had been to increase to overall yield of sophorolipids.

Table 1.2 Production and yields of sophorolipids from various authors.

Fermentation	Sophorolipids (g/L)	References
Batch	6.5 to 137	Albrecht *et al.* (1996), Asmer *et al.* (1988), Brakemeier *et al.* (1998), Casas and Garcia-Ochoa (1999), Cooper and Paddock, (1984), Deshpande and Daniels (1995), Garcia- Ochoa and Casas (1999), Gobbert *et al.* (1984), Ito and Inoue (1982), Ito *et al*. (1982), Klekner *et al*. (1991), Lee and Kim (1993), Zhou and Kosaric (1993 and 1995), Zhou *et al.* (1992)
Fed-batch	15 to 317	Asmer *et al.* (1988), Lee and Kim (1993), Brakemeier *et al.* (1995 and 1997), Davila *et al.* (1992 and 1997), Daniel *et al.* (1998a), Rau *et al.* (1996)
Continuous	94	Kim *et al.* (1997)
Resting Cells	15	Gobbert *et al.* (1984)
Two Stage	12 to 120	Daniel *et al.* (1998b and 1999), Otto *et al.* (1999)
Self-cycling	0.63	McCaffrey and Cooper (1995)

1.3.1 Batch fermentations

Batch fermentations of *C. bombicola* have been used as a convenient method of studying the nature of sophorolipid production. Moreover this approach involves the detailed study of some aspects of sophorolipid production by *C. bombicola* including the fermentation kinetics, alkane uptake, mass balances, and the production biochemical pathway. Authors have used batch reactions to study the fermentation kinetics of the sophorolipid production. Garcia-Ochoa and Casas (1999) proposed the use of an unstructured kinetic model for *C. bombicola* sophorolipid production under batch conditions. A medium containing glucose, food grade sunflower oil, and yeast extract was used. The authors based their model on three responses: biomass, glucose, and sophorolipids. It was assumed that the fatty acids from the vegetable oil were used for sophorolipids and the glycerol for maintenance energy. The model was fitted to experimental data by a non-linear algorithm in conjunction with a Runge-Kutta routine. The authors validated the model by fitting the model to their own data and other authors (Asmer *et al.*, 1988, Zhou *et al.*, 1992, Lee and Kim, 1993, Lang and Wagner, 1993). Garcia-Ochoa and Casas (1999) calculated various *C. bombicola* parameters including μ (0.14 h^{-1}), generation time (7.1 h), C_{xm} (6.0 g/L), μ_p (7.3 x 10^{-4} $g_P L/g_S g_X/h$), and $Y_{P/S}$ (0.59 g_P/g_S) with respect to glucose. These values did not account for the effect of vegetable oil, despite the use of glucose and vegetable oil for sophorolipid production.

Ito and Inoue (1982) showed that sophorolipids played a role in the uptake of alkanes in *C. bombicola*. The authors showed that sophorolipids stimulated the growth of *C. bombicola* on alkanes with 10 to 20 carbons, but had no effect on fatty alcohol, fatty

acids, glucose and glycerol uptake. The authors suggested that sophorolipids are involved in alkane dissimilation by *C. bombicola* through some undetermined mechanism. The authors also suggested that sophorolipids were specific growth factors (Inoue and Ito, 1982). Under the batch reaction conditions used by Lee and Kim (1993) a carbon balance showed that 13 %, 37 % and 50 % of carbon input from oil and glucose went into the biomass, sophorolipids and carbon dioxide, respectively.

Using glucose as a sole carbon source, Albrecht *et al.* (1996) studied the biochemical pathways involved in the initiation of sophorolipid biosynthesis. In that study phosphate limiting conditions were used to induce the production of sophorolipids in batch fermentations. These authors noticed a correlation between initiation of sophorolipid production and the decline of the specific activities of NAD and NADP dependent isocitrate dehydrogenase of the tricarboxylic acid cycle. They also showed that isocitrate and citrate accumulated in the mitochondria due to the constant citrate synthase activity. Effectors and inhibitors tested had no effect on the two previous enzymes. The authors concluded that the initiation of sophorolipid production was regulated at the level of enzyme synthesis and not enzyme activity.

1.3.2 Fed-batch

Various authors have studied fed-batch production of sophorolipids (Asmer *et al.*, 1988; Lee and Kim, 1993; Rau *et al.*, 1996; Daniel *et al.*, 1998a, 1998b; Davila *et al.*, 1992 and 1997). These reactions have been used mainly to increase yield of sophorolipids. Various feed rates were attempted to increase sophorolipid yields, however no consensus was

reached among different authors. The majority of authors, with the exception of Davila *et al.* (1997) used an arbitrary value for glucose and lipidic substrate feed rates. Davila *et al.* (1997) optimized the feeding rate of rapeseed ethyl esters to 1.75 mL/L/h and the glucose feed rate at greater than 0.6 g/L/h. Other authors used a wide range of lipidic and glucose substrate feed rates at 0.4 to 1.0 g/L/h and 0.6 to 2.0 g/L/h, respectively (Asmer *et al.*, 1988; Lee and Kim, 1993; Rau *et al.*, 1996; Daniel *et al.*, 1998a, 1998b).

In order to make comparisons between batch and fed-batch fermentations of different authors, the yield sophorolipids with respect to the consumption of oil and glucose was plotted (Figure 1.2). The highest amount of sophorolipids produced was obtained by fed-batch and was 317 g/L (Davila *et al.*, 1992 and 1997). In comparison the highest yield from batch was only 137 g/L. This figure also emphasized that fed-batch produced much higher levels of sophorolipids than the batch method. As indicated by several authors, the fermentation broth became extremely viscous at very high concentrations of sophorolipids. Moreover effective aeration and agitation became extremely difficult at high viscosity (Rau *et al.*, 1996; Daniel *et al.*, 1998a; Davila *et al.*, 1992 and 1997).

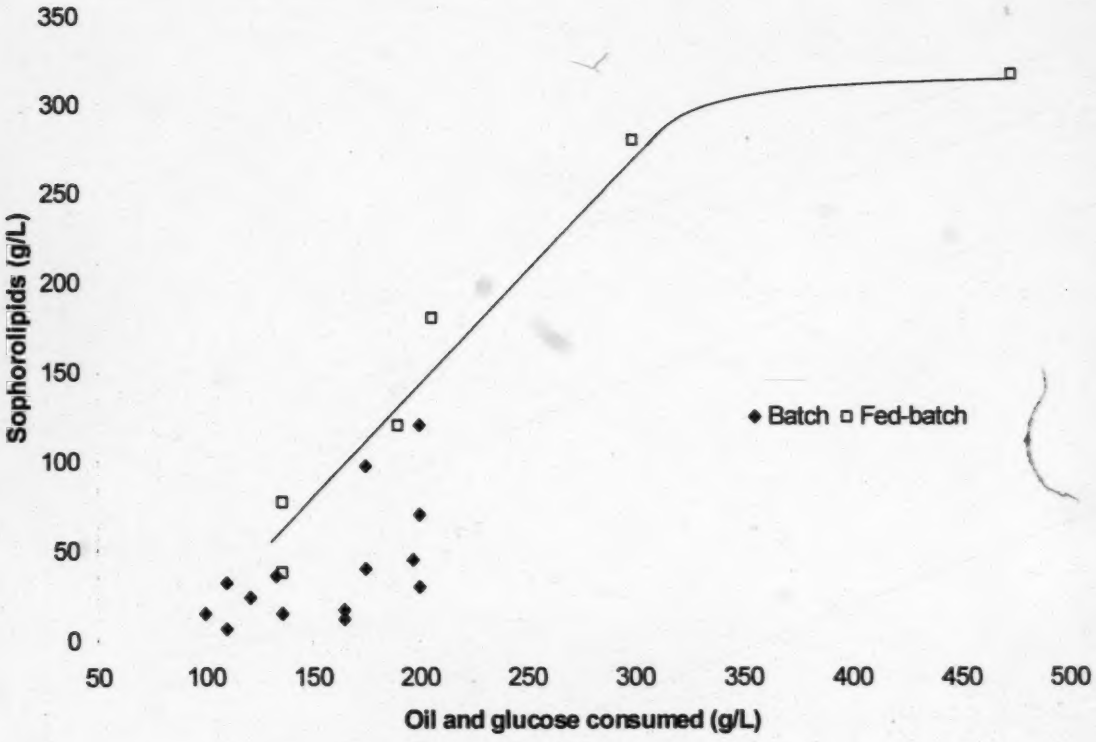

Figure 1.2 Production of sophorolipids from *C. bombicola.* Different authors conducted the batch and fed-batch fermentations. Lines are trends only. Batch fermentations: Albrecht *et al.* (1996), Asmer *et al.* (1988), Brakemeier *et al.* (1998), Casas and Garcia-Ochoa (1999), Cooper and Paddock (1984), Deshpande and Daniels (1995), Garcia-Ochoa and Casas (1999), Ito and Inoue (1982), Ito *et al.* (1980), Klekner *et al.* (1991), Lee and Kim (1993), Zhou and Kosaric (1993 and 1995), Zhou *et al.* (1992). Fed-batch fermentations: Asmer *et al.* (1988), Brakemeier *e t al.* (1995 a nd 1997), Daniel *e t al.* (1998a), Davila *et al.* (1992 and 1997), Lee and Kim (1993), Rau *et al.* (1996).

The efficiency of sophorolipid production can be analyzed by comparing the $Y_{P/S}$ and yield of sophorolipids. The maximum $Y_{P/S}$ was 0.93 with a sophorolipid concentration of 280 g/L obtained by Daniel *et al.* (1998a) (Figure 1.3). This figure also showed that the highest $Y_{P/S}$ did not directly lead to the highest amount of sophorolipids produced, because much higher yields of 315 g/L were obtained with $Y_{P/S}$ of 0.67. The study of Daniel *et al.* (1998a) differed from the others in that no glucidic substrate was used in the production of sophorolipids. Rapeseed oil was used as a main carbon source during growth of *C. bombicola* and production of sophorolipids. Secondly, the medium formulation was fount to contain very high concentrations of complex nitrogen in the form of cheese whey. The highest efficiency and product concentration did not occur in the same study. This finding indicated that in a fed-batch fermentation of sophorolipids a compromise must be made when deciding between high levels of sophorolipids or high efficiency.

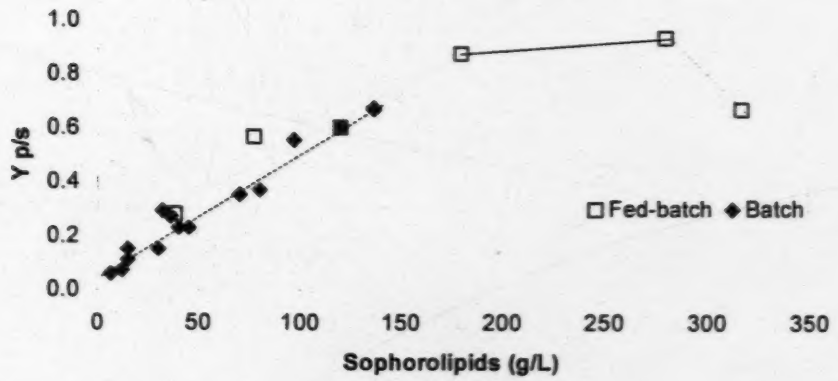

Figure 1.3 The $Y_{P/S}$ of batch and fed-batch fermentations of *C. bombicola.* The $Y_{P/S}$ was calculated from the previous figure. Lines are trends only.

1.3.3 Other methods of sophorolipid production

There are other techniques used to produce sophorolipids included resting cells (Gobbert *et al.*, 1984; Casas and Garcia-Ochoa, 1999), two stage fermentation (Daniel *et al.*, 1999; Otto *et al.*, 1999), continuous (Kim *et al.*, 1997) and self-cycling (McCaffrey and Cooper, 1995; Weber, 2001). The latter two methods of production are not discussed in this review. Gobbert *et al.* (1984) developed the resting cell method. This technique utilizes a culture of *C. bombicola* harvested during stationary phase. The authors have shown that the growth medium need not contain any lipidic substrates to stimulate the production of sophorolipids. However, the inclusion of a lipidic substrate in the growth medium would induce higher production of sophorolipids, because two carbon sources are favored for sophorolipid production (Cooper and Paddock, 1984). The spent medium was removed and the cells were re-suspended in a nutrient-free buffer at pH 3.5. Glucidic and lipidic carbon sources were then added to the culture to produce sophorolipids. Depending on the method of carbon addition, the resting cell method can fall into the typical batch, fed-batch or continuous production of sophorolipids. In continuous production, the cell culture could be used as catalysts for sophorolipid production. Casas and Garcia-Ochoa (1999) claimed to have used the resting cell method for sophorolipid production. However, the authors re-suspended the *C. bombicola* culture in a nutrient-containing medium instead of a nutrient-free buffer. This variation led to further growth of the cultures when glucose and sunflower oil were added in batch fermentations. Their study is more akin to using a high inoculum density as oppose to the resting cell method. This method produced the high yields of sophorolipids. Maximum sophorolipids (120 g/L) were obtained in 8 d with $Y_{P/S}$ of 0.60.

Daniel *et al.* (1999) developed the two-stage fermentation with *C. bombicola* and *Cryptococcus curvatus* in order to remove lactose from deproteinized whey. *C. curvatus* (ATCC 20509) is an oleaginous yeast capable of consuming lactose as a carbon source. This organism also produces single cell oil during the stationary phase. Deproteinized whey concentrate was used as the growth medium for *C. curvatus* fermentation during the first stage. The culture and single cell oil were then used as nutrient and carbon sources for *C. bombicola*. Glucose was added to increase the amount of sophorolipids produced to 12 g/L (Daniel *et al.*, 1999). The deproteinized whey was obtained from the cheese whey wastewater after proteins of commercial interest such as lactalbumin and lactoferrin were removed. Approximately 70 % of lactose was further removed from the whey by crystallization. After these steps, the deproteinized whey still contained lactose 50 g/L (Daniel *et al.*, 1999). Zhou and Kosaric (1993, 1995) were able to produce sophorolipids from *C. bombicola* grown in medium containing deproteinized whey. However, Daniel *et al.* (1999) discovered that lactose was not consumed during the fermentation of *C. bombicola*. As a result the problem of lactose in the wastewater, could not be eliminated by the method of Zhou and Kosaric (1993, 1995).

1.4 Sophorolipid production conditions

1.4.1 Medium composition

Both glucidic and lipidic carbon sources are essential for the over production of sophorolipids (Cooper and Paddock, 1984). Very low quantities of sophorolipids are produced when a lipidic carbon source is not used (Gobbert *et al.*, 1984). The fatty acid tail of the sophorolipids is created *de novo* when glucose is used as the only substrate for

production (Albrecht *et al.*, 1996). In comparison the majority of lipidic substrates are directly incorporated into the fatty acid tail of the sophorolipids (Spencer *et al.*, 1979). Glucidic substrates that have been used in the cultivation of *C. bombicola* include glucose, sucrose, lactose, fructose, and mannose (Klekner *et al.*, 1991; Zhou and Kosaric, 1993; Gobbert *et al.*, 1984). Controversy surrounds the ability of *C. bombicola* to consume lactose. Zhou and Kosaric (1993 and 1995) were able to produce sophorolipids using lactose as the sole glucidic source. *C. bombicola* did not consume any lactose when sophorolipids were produced using medium containing deproteinized whey concentrate and lactose100 g/L (Daniel *et al.*, 1998). Another study also showed that *C. bombicola* cultures were unable to consume lactose (Spencer *et al.*, 1970).

The overproduction of sophorolipids tended to be favored by the inclusion of natural oils from plants and animals. Lipidic substrates that have been used in the batch production of sophorolipids include vegetable oils from canola, safflower, soybean, and sunflower (Zhou and Kosaric, 1993; Ito and Inoue, 1980, Ito *et al.*, 1982; Zhou *et al.*, 1992; Gobbert *et al.*, 1984; Lee and Kim, 1993; Cooper and Paddock, 1984; Klekner *et al.*, 1991; Casas and Garcia-Ochoa, 1999; Garcia-Ochoa and Casas, 1999). Animal fats have also been shown to be effective substrates for sophorolipid production (Deshpande and Daniels, 1995). Lipidic substrates used in fed-batch cultures were soybean oil, rapeseed oil, and rapeseed oil ethyl esters have been used (Rau *et al.*, 1996; Lee and Kim, 1993; Daniel *et al.*, 1998a, 1998b; Davila *et al.*, 1992, 1997). Oleic acid has also been used for overproduction of sophorolipids using both batch and fed-batch fermentation (Asmer *et al.*, 1988; Rau *et al.*, 1996).

Individual fatty acids, hydrocarbons and defined mixtures thereof have been used to test the feasibility of producing novel sophorolipids. The incorporation of fatty acids from palmitic to docosanoic acids (16 to 20 carbons) into sophorolipids was indirectly observed. A similar study was accomplished with hydrocarbons from tetradecane to docosane (14 to 20 carbons) (Tulloch *et al.*, 1962; Spencer *et al.*, 1979; Cavalero, 2001). Unfortunately, no structures were shown in the previous studies. Alcohols such as 1-dodecanol, 2-dodecanol, 2-tetradecanol, 2-hexadecanol, 2,13-tetradecandiol, and 2,15-hexadecandiol have all been successfully incorporated into sophorolipids (Brakemeier *et al.*, 1995; Brakemeier *et al.*, 1997; Lang *et al.*, 1995). Ketones such as 2-, 3- and 4-dodecanones have also been incorporated into sophorolipids (Brakemeier *et al.*, 1998).

The nitrogen sources are chosen based on their low cost and novelty. The most common organic nitrogen source is yeast extract (Zhou *et al.*, 1992, Zhou and Kosaric, 1993; Lee and Kim, 1993; Cooper and Paddock, 1984; Casas and Garcia-Ochoa, 1999; Klekner *et al.*, 1991). A single organic nitrogen source is often used in the production medium (Cooper and Paddock, 1984; Garcia-Ochoa and Casas, 1999; Klekner *et al.*, 1991). Other sources of organic nitrogen are corn steep liquor and cheese whey from dairy wastes (Deshpande and Daniels, 1995; Zhou and Kosaric, 1993). Several authors have used inorganic nitrogen sources such as urea or ammonium in addition to the organic nitrogen (Zhou *et al.*, 1992; Zhou and Kosaric, 1993; Deshpande and Daniels, 1995; Lee and Kim, 1993). All of these authors, with the exception of Casas and Garcia-Ochoa (1999), have

included other minerals in the production medium such as iron, magnesium, potassium, calcium, sodium, chloride, phosphate, and sulfate.

1.4.2 Parameters for fermentation control

In order to maximize the production of sophorolipids several authors have manipulated the temperature, pH and dissolved oxygen concentration of the growth and production conditions of batch reactions. The growth of *C. bombicola* around 22 °C to 25 °C has been shown to produce the highest sophorolipids while using glucose and tall-oil fatty acids. Product formation rapidly decreased at temperatures above 30 °C (Spencer *et al.*, 1979). Gobbert *et al.* (1984) showed that optimum sophorolipid production for resting cells was at 21 °C. When *C. bombicola* batch cultures were grown at 25 °C and temperature was increased to 37 °C during the production phase, sophorolipids production was reduced from 317 g/L to 25 g/L (Davila *et al.* 1997). Nonetheless, it is still common practice to produce sophorolipids at 30 °C, because temperatures below 25 °C result in large cooling costs in industrial situation. A second drawback of lower temperatures is that some fats and oils solidify below 25 °C (Deshpande and Daniels, 1995).

The pH of a typical batch fermentation of *C. bombicola* drops from the initial medium pH 7.0 to 3.0 after 24 h of incubation. Deshpande and Daniels (1995) were able to increase the yield from 100 g/L to 120 g/L by maintaining the pH at 3.4 after 24 h, while reducing the maximum time of fermentation from 72 h to 68 h. Resting cells of *C. bombicola* at pH 3.5 showed the highest sophorolipids production when glucose was used as a carbon

source (Gobbert *et al.*, 1984). Using glucose and rapeseed oil esters in fed-batch Davila *et al.* (1997) showed that pH 3.5 was also optimal for sophorolipids production. An increase in pH from 3.5 to 5.0 decreased sophorolipids production from 317 g/L to 190 g/L. Increasing the pH from 5.0 to 8.0 led to cell lysis (Davila *et al.*, 1997). It appears that production of sophorolipids is favored at pH 3.5 for batch and resting cell conditions.

The amount of molecular oxygen in the liquid medium during sophorolipid production is significant. The oxygen atom used to hydroxylate the last or the penultimate carbon of the eighteen-carbon fatty acid used in sophorolipid production is obtained from the dissolved oxygen in the medium. Heinz *et al.* (1969) showed that ^{18}O-labeled molecular oxygen and not ^{18}O-labeled water was incorporated in the hydroxylated fatty acids of sophorolipids. With this in mind the rate of aeration in sophorolipid fermentation can greatly affect the amount of sophorolipids produced. Shaker speeds of 200 rpm to 450 rpm have been sufficient for sophorolipid production (Spencer *et al.*, 1979). For stirred tank fermenters 0.5 v/v/min to 2.0 v/v/min are sufficient for sophorolipids production (Deshpande and Daniels, 1995; Zhou and Kosaric, 1995).

1.4.3 Optimization of media formulations

The main objective in the creation of different media and their optimization has been to increase the yield of sophorolipids. Batch reaction is a convenient method for studying the effects of the production medium on the yield of sophorolipids. The chosen batch reactors for these studies are the shake flask and stirred tank batch reactor. In the growth medium, certain parameters are optimized to produce the highest density of *C. bombicola*

in the least amount of time. Deshpande and Daniels (1995) optimized six parameters including glucose (1.5 % m/v), animal fat (0.5 % v/v), corn steep liquor (0.5 % m/v), urea (0.1 % m/v), and potassium phosphate (0.2% m/v). The maximum OD observed was 28.5 after 24 h of growth. Casas *et al.* (1997) optimized four parameters including glucose (1.2 % m/v), ammonium chloride (0.19 % m/v), potassium phosphate (0.74 % m/v), and magnesium chloride (0.02 % m/v). The authors of this study chose to keep several other medium parameters constant. Glucose and phosphate had the greatest effect on the biomass density. The glucose consumption rate was less at concentrations above 1.2 % (m/v) and required more than 30 h for complete consumption. In comparison cell density at 30 h increased when glucose concentration was increased to 1.2 % (m/v). Thus glucose at 1.2 % (m/v) with complete consumption in 30 h was optimal.

The optimization of the production medium generally involved altering a maximum of three variables including oil, glucose, and yeast extract. The ratio between each variable is optimized to produce the highest concentrations of sophorolipids. It should be noted that each study was independent of the other and the optimum production medium was achieved from the manipulation of the three said variables within the authors' medium formulation. All authors reached a common conclusion of using an m/m of 1 for oil and glucose, despite the use of different types of vegetable oils. The concentration of oil (v/v) and glucose (m/v) corresponded to approximately 10 % of the production medium. In contrast, no convergence seemed to have emerged between the concentrations of yeast extract. Within each study the authors varied the concentrations of yeast extract from 0 % (m/v) to 2.0 % (m/v) and chose the optimum concentration. Zhou *et al.* (1992) were the

most successful of the group, because their chosen concentration of yeast extract at 0.25 % (m/v) led to the overall highest sophorolipids yield of 13.7 % (m/v). A summary of the optimum ratios using this approach is shown in Table 1.3.

Table 1.3 Summary of the optimization of the production medium by several authors. Compositions of oil and others are % v/v and % m/v, respectively.

Oil type	Oil	Glucose	Yeast extract	Sophorolipids	Reference
Sunflower	10	10	0.1	4.0	Casas and Garcia-Ochoa, 1999
Safflower	10.5	10	0.25	13.7	Zhou *et al.*, 1992
Canola	10	10.5	0.4	8.0	Zhou and Kosaric, 1995
Safflower	10	10	0.5	7.0	Cooper and Paddock, 1984
Sunflower	9.5	10	1.0	4.5	Klekner *et al.*, 1991

2.0 Motivation and objective of thesis

The overall motivation for this thesis was to increase the commercial application of sophorolipids produced by *C. bombicola*. The trend in the production of sophorolipids has been to increase the yields of production. As indicated in Table 1.2 several methods of production exist and they show varying degrees of successfulness. However, none have focused on the purity of sophorolipids during these fermentations. The marketability of sophorolipids can further benefit from an increase in purity.

The objective of this thesis was to analyze the purity of sophorolipids. More specifically, the purity of sophorolipids and the composition changes of the sunflower oil fatty acids were analyzed during sophorolipid production. Other lipid products were observed within

the sophorolipids. The composition and chemical structure of these lipid products were determined. The length of fermentation time required to establish a batch of sophorolipids with high purity was established. *C. bombicola* was cultivated in batch conditions. The medium formulation used to achieve this was sunflower oil, glucose, yeast extract, and water. This medium was chosen for its few ingredients and ability to produce high production of sophorolipids.

3.0 Materials and Methods

3.1 Culture cultivation

D. G. Cooper (McGill University, Montreal, Canada) kindly provided the *C. bombicola* (ATCC 22214) culture used in this work. Unless stated otherwise, all media components used were from the Difco brand (BD Diagnostic Systems, Sparks, US), all fine chemicals were obtained from Sigma-Aldrich (Poole, UK), and all glassware was obtained from Fisher Scientific (Leicestershire, UK). Reverse osmosis water was used in all aqueous solutions.

The *C. bombicola* cultures were transferred monthly in an YM plate agar: 2.1 % (m/v) YM and 1.5 % (m/v) technical agar. The YM agar plates were grown for 3 d at room temperature and stored at 4 °C. One loop of culture from the agar plate was used to inoculate the liquid growth medium. The liquid inoculum medium contained 2.1 % (m/v) YM broth (100 mL in 500 mL shake flask). Liquid cultures were grown in an incubator shaker (Innova shaker model 4300, New Brunswick Scientific, Edison, US) for 32 h at 30 °C and 200 rpm. The cell culture was harvested at late-log phase with a corresponding

OD of 10 ± 1.0, and 10 % (v/v) of the culture was then used to inoculate the production medium: 10 % (m/v) glucose, 10 % (v/v) food-grade sunflower oil (Canbra Foods Limited, Lethbridge, Canada), and 0.1 % (m/v) yeast extract. These cultures were grown in 100 mL of liquid medium in 500 mL shake flasks for 0 d to 20 d under similar conditions as the inoculum culture.

3.2 Biomass determination

The cell density of *C. bombicola* cultures was determined by two methods. The first method used was OD at 650 nm using the UV-visible recording spectrophotometer (Shimadzu Corporation, Kyoto, Japan). For consistent readings, it was necessary to let the shake flask culture settle for 2 min. This allowed the oil, water and sophorolipid phases to separate. A sample of 500 µL was taken from the middle water phase making sure not to disturb the oil and sophorolipid phases. The second method used was DCM. The DCM was determined after the sophorolipid producing cultures were washed with hexane followed by ethyl acetate. Samples of 5 mL were removed from the aqueous phase and centrifuged at maximum speed for 5 min in the clinical centrifuge (IEC, Needham Heights, US). The pellet was dried at 80 °C for 24 h and mass determined gravimetrically.

3.3 Sunflower oil and sophorolipid extraction

This method was adapted from Ito *et al.* (1980). All organic and aqueous solvents were used at room temperature. The culture broth was extracted thrice with equal volumes of hexane in order to remove residual vegetable oil. The organic phase was retained for

further analysis. The culture broth was then extracted thrice with equal volumes of ethyl acetate. The ethyl acetate phase was retained for further analysis. Each solvent extraction lasted for 5 min. Crude sophorolipids were obtained after evaporation of ethyl acetate in a rotating evaporator at 40 °C (model B-480 and R-114, Buchi, Flawil, Switzerland) under reduced pressure. A water aspirator was used to reduce pressure to 20 kPa. Prior to filtration (qualitative filter #5, Whatman, Cambridge, UK), the sophorolipid suspension in ethyl acetate extract was mixed with anhydrous $MgSO_4$ to remove the water. The mass of the crude sophorolipids were determined gravimetrically and stored in a dessicator at room temperature. The spent culture broth was allowed to settle for 24 h and the residual sophorolipid phase was drained, lyophilized and retained for further analysis.

3.4 LC of sophorolipids

This method was adapted from Deshpande and Daniels (1995). The glass column used had an internal diameter of 2.5 cm, height of 76 cm and PTFE stopcock. The column was packed with glass wool and sand (height of 2 cm). Silica gel 50 g (Kieselgel 60, Fluka, Poole, UK) was suspended in hexane and loaded into the column. The hexane was drained to 0.5 cm above the silica gel. The column was loaded with 150 mL of chloroform and drained to replace the hexane phase. The 20 d sophorolipid mixture (1 g) was dissolved in ethyl acetate (5 mL) and loaded into the column. Fractions were eluted from the column by subsequent washes of 0 %, 5 %, 10 %, 15 %, and 50 % (all v/v) of methanol/chloroform at 150 mL/wash. A total of 6 consecutive runs were used to separate 6 g of sophorolipids, and similar fractions were pooled together. The solvent from each fraction was evaporated using the same method previously described.

3.5 Lyophilization (freeze drying) of sophorolipids

After ethyl acetate extraction, the residual sophorolipids in the production medium were shell frozen in a bath of dry ice and ethanol. The sophorolipids were lyophilized in the Freeze Mobile 24 (Virtis Company, Gardiner, US). Dried sophorolipids were stored in a dessicator at room temperature.

3.6 TLC of sophorolipids

This method was adapted from Asmer *et al.* (1988). Dried sophorolipids in ethyl acetate 10% (m/v) were made and 1 drop of acetic acid (using a Pasteur pipette) was added to each solution. Using a capillary tube, 1 µL was spotted on each lane of the TLC silica gel (20 cm x 20 cm and 250 µm layer, Whatman, Cambridge, UK). The plates were developed for 15 min using the eluting solvent: chloroform, methanol and water with a ratio of 65:15:2 (v/v/v). The TLC plates were allowed to dry at room temperature for 5 min. The detecting reagent used was glacial acetic acid, sulfuric acid, *p*-anisaldehyde with a ratio of 100:2:1 (v/v/v) and sprayed onto the TLC plates. Sprayed plates were heated at 125 °C (isotemp vacuum oven, model 281A, Fisher Scientific, Leicestershire, UK) for 5 min. Sophorolipids appeared as dark spots, while other lipids and free fatty acids were light spots.

TLC analysis of the crude sophorolipids produced a spot labeled "G" immediately above the corresponding spot for di-acetylated lactone. The content of the TLC spot "G" was determined. Sophorolipids from 15 d of fermentation were separated using a preparative

TLC plate (20 cm x 20 cm and 1 mm layer, Whatman, Cambridge, UK). The contents of the spot from the TLC was scraped and extracted thrice with a total of 6 mL of ethyl acetate. The ethyl acetate was evaporated and the contents were converted to FAME.

3.7 Alkaline hydrolysis (saponification) of sophorolipids

This method was adapted from Rau *et al.* (1999). Crude sophorolipid mixtures were saponified to obtain a large quantity of de-acetylated acidic sophorolipids. A 40 % suspension of sophorolipids was made in 100 mL of 5 M NaOH. The sophorolipids were suspended using a mild sonicating bath (Bransonic model 52, Branson Ultrasonic Corporation, Danbury, US) for 25 min. The sophorolipid suspension was then refluxed at 98 °C for 24 h in a glove heater (Glas-Col, Terre Haute, US) with a variable autotransformer (Staco Energy Products Company, Dayton, US). The temperature was monitored by a thermocouple (model 52, Supelco, Bellefonte, US).

The saponified sophorolipids were cooled to room temperature in a water bath and titrated with HCl to pH 4.0. An acidic pH was necessary to ensure the acidic sophorolipids were protonated. The aqueous phase was washed thrice with equal volumes of 1-pentanol. Acidic sophorolipids were obtained after evaporation of 1-pentanol in a water bath at 60 °C (model B-480 and R-114, Buchi, Flawil, Switzerland) under reduced pressure. A direct drive high vacuum pump (Marvac Scientific Company, Concord, US) was used to reduce pressure to 15 kPa. Residual 1-pentanol was further evaporated for 24 h in a vacuum oven (isotemp vacuum oven, model 281 A, Fisher Scientific, Leicestershire, UK) at 50 °C and 10 kPa.

The precipitate was suspended in 50 % (v/v) methanol/chloroform and centrifuged for 5 min in the clinical centrifuge (IEC, Needham Heights, US). Centrifugation was necessary to remove sodium chloride salts from the acidic sophorolipids. The 50 % (v/v) methanol/chloroform was then evaporated using the same set up as stated in the crude sophorolipid extraction.

3.8 FAME analysis of sunflower oil and contents of TLC spot "G"

The sunflower oil and hexane extracted sunflower oil were converted to FAME. The methylating agent used was 2 % sodium methoxide in methanol mixed with Skellysolve B (3/1, v/v) (Hougen *et al.*, 1973; Freedman *et al.*, 1986; Bannon *et al.*, 1982). The reaction time was 30 min at 25 °C. The solution was washed with distilled water (1 mL). The organic phase was injected into the gas chromatograph (model Sigma 3B, Perkin-Elmer, Courtaboeuf, France) using a fused silica column (wax 10, 30 m x 0.53 mm, 1.0 μm coating, Supelco, Bellefonte, US).

The method used to analyze the FAME from the contents of the TLC spot was adapted from Bressler and Fedorak (2001). The content of the TLC spot "G" was converted into FAME using diazomethane as the methylating agent. The contents were analyzed by GC-MS using a Hewlett Packard 5890 series II GC with a 5970 series mass detector and a 30 m DB-5 capillary column (J-W Scientific, Folsom, US). The GC temperature program used was 90 °C for 1 min and then 5 °C/min to 280 °C for 21 min (Bressler and Fedorak, 2001).

3.9 Surface and interfacial tension of acidic sophorolipids

Surface and interfacial tension measurements were made with a manual tension meter (Model 70545, Central Scientific Company, Franklin Park, US). A platinum ring (Model 70542, Central Scientific Company, Franklin Park, US) was used to measure the tension as the ring passed through the liquid-liquid and liquid-air interfaces. Measurements were made at room temperature between 22 °C and 24 °C. Reverse osmosis water (20 mL) and toluene (20 mL) were used for the aqueous and organic solutions respectively. The 100 mL beakers used for all measurements were soaked overnight in soapy water (Sparkleen, Fisher Scientific, Leicestershire, UK). All beakers were rinsed thrice in reverse osmosis water and air-dried. The measured surface and interfacial tension of reverse osmosis water (73 mN/m) and water-toluene (36 mN/m) were used as references for the cleanliness of the beakers. The acid sophorolipids were dissolved in water and toluene for the surface and interfacial tension measurements respectively. Serial dilutions of different concentrations of these solutions were made prior to the measurements.

4.0 Results

4.1 Biomass and substrate determination

Two methods exist for measuring biomass of *C. bombicola* namely OD and DCM. The effect of solvent extraction on biomass was tested to determine the feasibility of using DCM to measure biomass. *C. bombicola* cultures were grown in YM broth for 3 d (Figure 4.1). The medium formulation and the length of fermentation did not lead to overproduction of sophorolipids. It was important to use conditions that did not lead to sophorolipid overproduction in order to accurately determine the effect of hexane and ethyl acetate extractions on biomass measurements. In the fermentation with *C. bombicola*, hexane was used to extract the vegetable oil from the liquid broth, followed by ethyl acetate to extract the sophorolipids. The DCM was determined before and after subjecting the cultures for oil and sophorolipid extraction. The data showed that the DCM reduced considerably after treatment with solvents (Figure 4.1). It should be noted the y-axis in this figure was plotted on an arithmetic scale to emphasize the differences in DCM. The ratio of DCM reduction was only constant during the stationary phase. Hexane and ethyl acetate were tested separately to determine which solvent had the most effect on the DCM. The analysis showed that ethyl acetate led to more significant reduction in DCM than hexane (Table 4.1). OD was used instead of DCM to measure biomass due to the detrimental effect of solvent extraction on biomass.

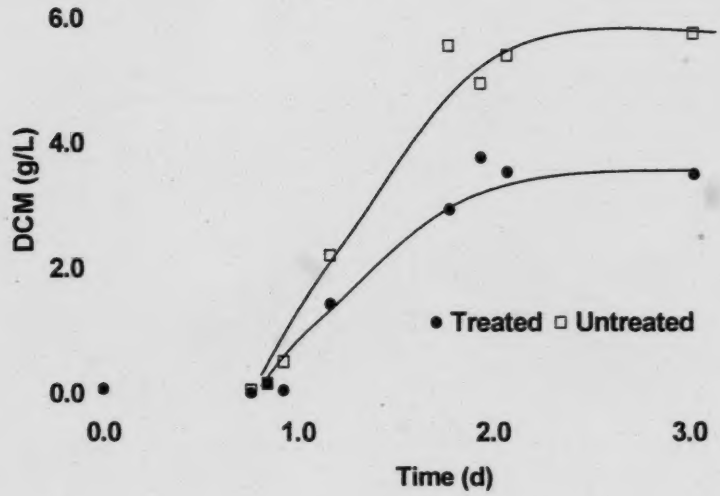

Figure 4.1 DCM determination of *C. bombicola* grown in YM broth. Samples labeled "treated" were subjected to hexane and ethyl acetate extractions, whereas samples labeled "untreated" were not subjected to these solvents. Lines are trends only.

Table 4.1 Effect of hexane and ethyl acetate extractions on the measurement of DCM of *C. bombicola*. A total of five samples were used for each extraction.

Extraction method	DCM (% m/v)	Deviation (±)
None	100	1
Hexane only	98	2
Ethyl acetate only	66	6

Sunflower oil was used as the lipidic substrate for the production of sophorolipids. The sunflower oil was converted to FAME and analyzed for fatty acid composition during the production of sophorolipids. Sodium methoxide in methanol was used as the methylating agent. The FAME were subjected to GC and the concentration of each fatty acid was calculated from the total mass of sunflower oil. The majority of the oil was composed of (in increasing amounts) 18:2, 18:1, 16:0, and 18:0 fatty acids (Figure 4.2). It should be noted that the composition error was 0.01 %, and error bars were not observable. The remaining fatty acids constituted less than 5 % of the total (Figure 4.3).

The effect of hexane extraction on the composition of fatty acids within the sunflower oil was analyzed to determine any discrepancy with the extraction of oil on its composition. A 10% (v/v) heterogeneous mixture of sunflower oil with water was created to mimic the actual fermentation environment. The oil was extracted thrice with hexane at equal volumes to the aqueous phase. GC analysis showed that the effect of hexane on composition of the four major fatty acids was minimal (Figure 4.2). The order of magnitude of the four major fatty acids was unaffected by hexane extraction of the oil. The hexane extraction had an effect on five of the minor fatty acids, namely 16:1, 20:1, 20:2, 22:0, and 22:1 fatty acids (Figure 4.3). The hexane extract showed higher values for 16:1 and 20:1 than expected. In comparison lower values were observed for 20:2, 22:0 and 22:1 than expected.

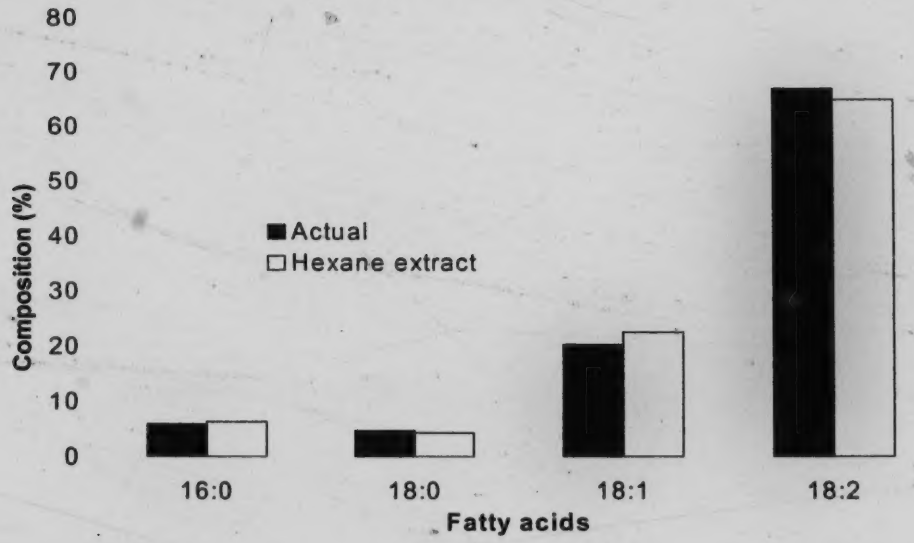

Figure 4.2 Major fatty acids of sunflower oil used in the production of sophorolipids from *C. bombicola*.

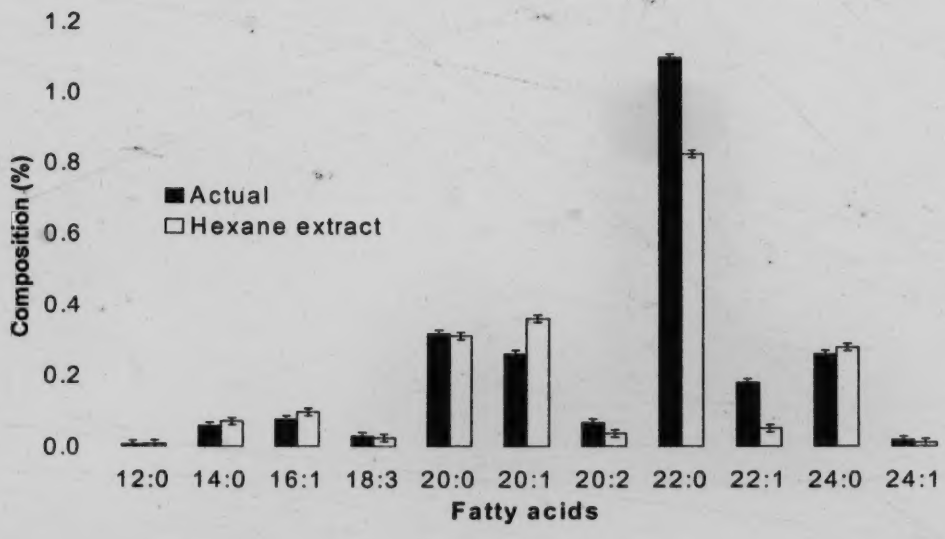

Figure 4.3 Minor fatty acids of sunflower oil used in the production of sophorolipids from *C. bombicola*.

4.2 Fermentation of *C. bombicola*

C. bombicola was grown in the inoculum medium to determine the ideal time for transfer into the sophorolipid production medium. The ideal time for transfer was set for the late exponential phase. One loop of *C. bombicola* was transferred from the solid medium into the inoculum broth, both containing YM. The cultivation temperature and agitation were 30 °C and 200 rpm, respectively. The cultures of *C. bombicola* were at exponential phase shortly after 0 d to 1 d (Figure 4.4). The late exponential phase was found to occur after 1.5 d of cultivation and corresponded to an OD of 10 (Figure 4.4). The maximum OD was approximately 20 and occurred after 2 d of growth. Overall the pH was reduced from 6.00 to 3.75 during the fermentation and slightly increased to 4.25 near the end of fermentation. A 10 % (v/v) cell culture was chosen for transfer into the production medium.

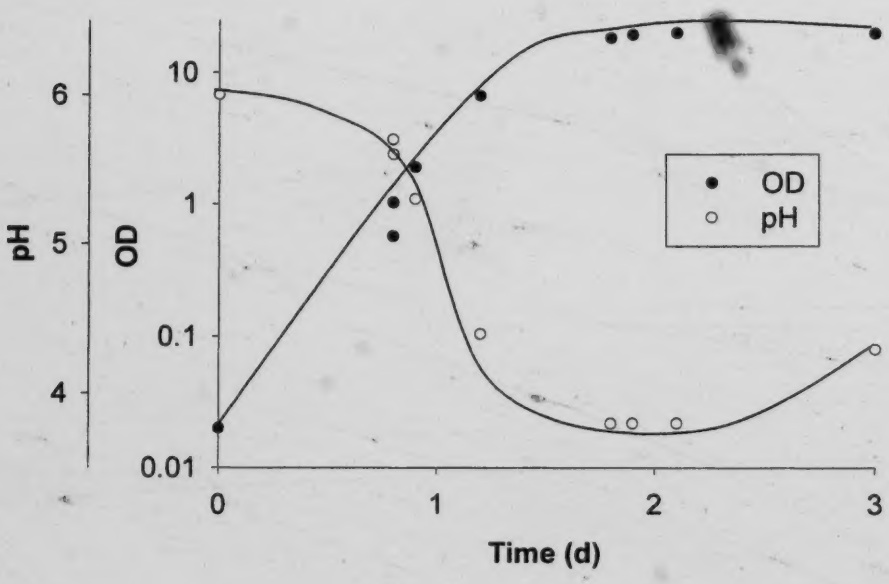

Figure 4.4 Growth of *C. bombicola* in batch fermentation containing inoculum medium. Lines are trends only.

An inoculum transfer scheme was developed to ensure consistent and high production of sophorolipids. One streak of cell colonies from the YM agar plate was transferred into the YM broth (inoculum medium). After 36 h, 10 % (v/v) of the cell culture in the inoculum medium (OD of 10) was transferred into 100 mL/flask of the production medium containing yeast extract, glucose and sunflower oil. The cell cultures were grown in shake flasks for 0 d to 20 d. The cultivation temperature and agitation were similar for the inoculum and production cultures.

In the production medium, *C. bombicola* cultures grew rapidly from 0 d to 1 d, and the lag phase was less than the first sampling time (Figure 4.5a). OD increased from 1 d to 8 d at a slower rate than those observed from 0 d to 1 d. The cell cultures were in the stationary phase from 8 d to15 d, and OD decreased from 15 d to 20 d of fermentation. The corresponding pH decreased rapidly from 6.0 to 2.5 from 0 d to 2 d and remained at pH 2.5 (Figure 4.5a).

The production and consumption profiles of sophorolipids and oil were very similar (Figure 4.5b). Both profiles were nearly linear from 0 d to 8 d, and both have the highest rates at these time intervals. The rate of consumption and production decreased from 8 d to 20 d. The sunflower oil was completely consumed after 20 d of fermentation leading to a maximum sophorolipids production of 45 g/L (Figure 4.5b). It should be noted that the minimum concentration of sunflower detectable was 0.1 g/L.

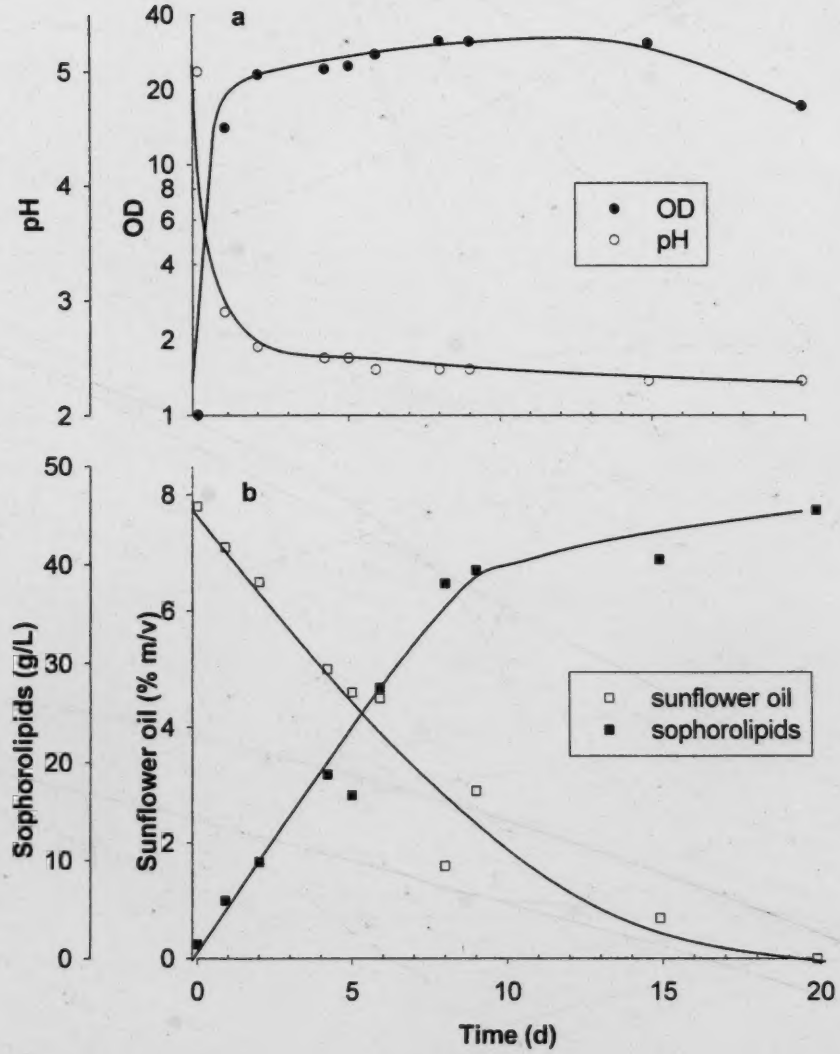

Figure 4.5 Growth of *C. bombicola* (a) and production of sophorolipids (b) in batch fermentation containing production medium with sunflower oil. Lines are trends only.

The fatty acid composition of sunflower oil was analyzed during the fermentation of *C. bombicola* in the production medium. The objective of the analysis was to determine if some were preferentially consumed over others. Fatty acids were not analyzed after 20 d of fermentation, because the hexane extraction yield was less than 0.1 g/L. The fatty acids 12:0, 14:0, 16:1, and 20:2 were not analyzed, because the overall concentration of each was less than 0.1 g/L. Each fatty acid concentration was normalized in order to compare the rate of consumption. The concentration of fatty acids 18:2, 18:1, 16:0, 18:0, 20:1, and 18:3 were normalized with respect to the initial concentration. The concentration of minor fatty acids 20:0, 22:0, 22:1, and 24:0 were normalized with respect to the maximum concentration of each, because values were detected above the initial concentration.

The order of the fatty acid consumption rates (lowest to highest) was 18:2, 18:1, 16:0, 18:0, 20:1, and 18:3 (Figure 4.6). These fatty acids were equally consumed after 15 d, and their concentrations after 20 d were below the detectable limit of 0.1 g/L. The consumption profiles of 16:0 and 18:1 fatty acids were very similar. The initial consumption rates of 18:2, 18:1, 16:0, 18:0, 20:1, and 18:3 fatty acids were reflective of their initial composition within the sunflower oil. More specifically, the order of initial composition values of fatty acids within the sunflower oil (highest to lowest) was 18:2, 18:1, 16:0, 18 :0, 20:1, and 18:3. Thus, the most abundant fatty acid (18:2) had the lowest initial rate of consumption, while the least abundant fatty acid (18:3) had the highest initial rate of consumption.

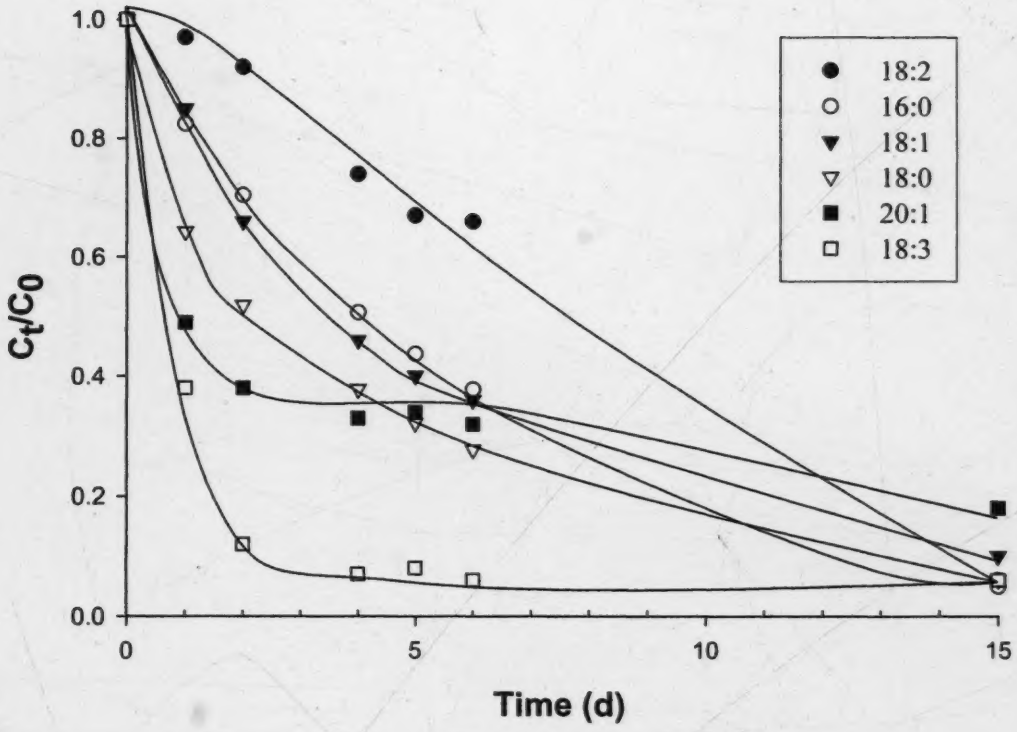

Figure 4.6 Consumption of sunflower oil fatty acids during batch fermentation of *C. bombicola* in the production medium. Lines are trends only.

The consumption profiles of minor 20:0, 22:0, 22:1, and 24:0 fatty acids fluctuated during the production of sophorolipids (Figure 4.7). Overall, the concentrations of 20:0 and 22:1 fatty acids decreased from their initial values after 15 d despite the overall scatter. The concentration of 22:0 fatty acid increased to twice the initial value after 6 d, only to decrease to the initial value after 15 d. The 24:0 fatty acid increased considerably from 0 d to 15 d.

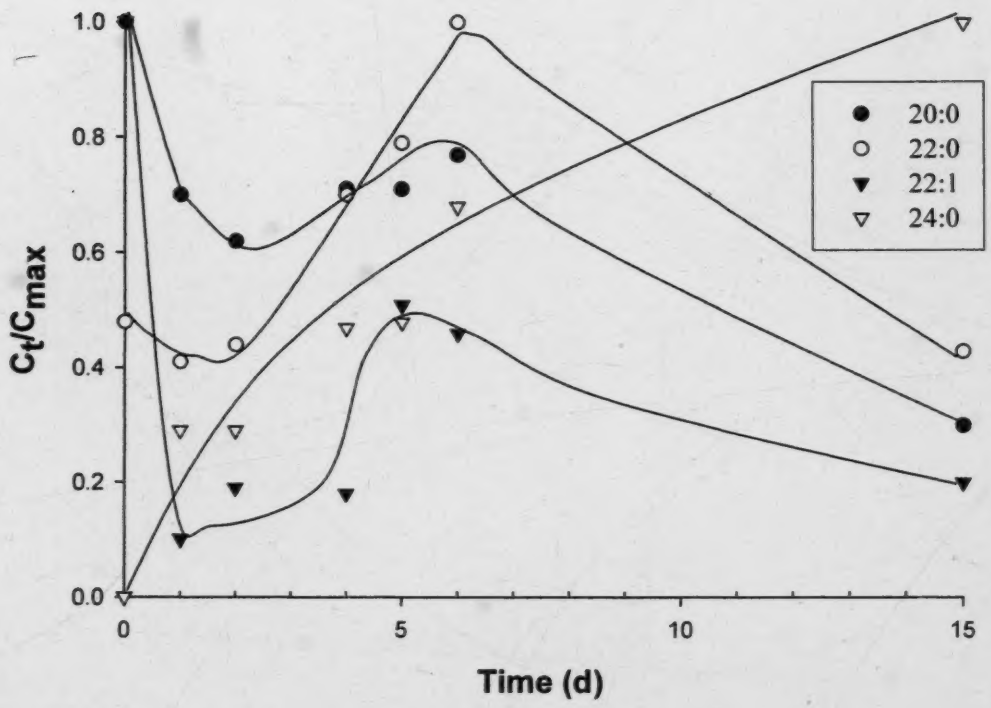

Figure 4.7 Consumption some minor fatty acids of sunflower oil during batch fermentation of *C. bombicola* in the production medium. Lines are trends only.

4.3 Sophorolipid recovery and characterization

4.3.1 TLC and LC of sophorolipids

C. bombicola was grown in the production medium for 20 d to study the nature of sophorolipid production. TLC was used to analyze the composition of the sophorolipids during the fermentation. It should be noted that each sample on the TLC corresponds to an initial load of 50 μg. A sample load less than 50 μg was not detectable, while greater than 50 μg led to over loading of the TLC plate. The R_f of the spots was not absolute and the values tended to float depending on the length of separation. The spots A to D and E to F (Figure 4.8) corresponded to lactones and acidic sophorolipids, respectively. The structures of A, B, C, D, E, and F have been previously determined, and corresponded to diacetylated lactone, 6"-acetylated lactone, non-acetylated lactone, a mixture of lactones with the ester bond at the 6'or 6" positions, 6'-acetylated acidic sophorolipids, and non-acetylated acidic sophorolipids (Asmer *et al.*, 1988). The composition of sophorolipids did not change dramatically from 2 d to 15 d of fermentation. The spot "G" was present within the sophorolipid mixture during days 2 d to 15 d of fermentation (Figure 4.8). This indicated that the sophorolipid mixture contained other impurities during the fermentation. This spot was not detected after 20 d of fermentation leading to a purer mixture of sophorolipids.

After four ethyl acetate extractions, a sophorolipid phase was still present at the bottom of the separatory flask. A total of four extractions with ethyl acetate did not remove this phase. The remaining sophorolipid phase was allowed to settle, lyophilized and measured gravimetrically. The amount of this phase was approximately 25 % of the sophorolipids

extracted by ethyl acetate. TLC was used to analyze the composition of lyophilized and ethyl acetate extracted sophorolipids (Figure 4.9). The intensity of the spots provided a clue to the composition of the lyophilized sample. The TLC of this sample showed that some spots had the same R_f values as lactones B to D. Moreover a large proportion of this had similar R_f values to acidic sophorolipids E and F. TLC did not separate an e ven larger proportion.

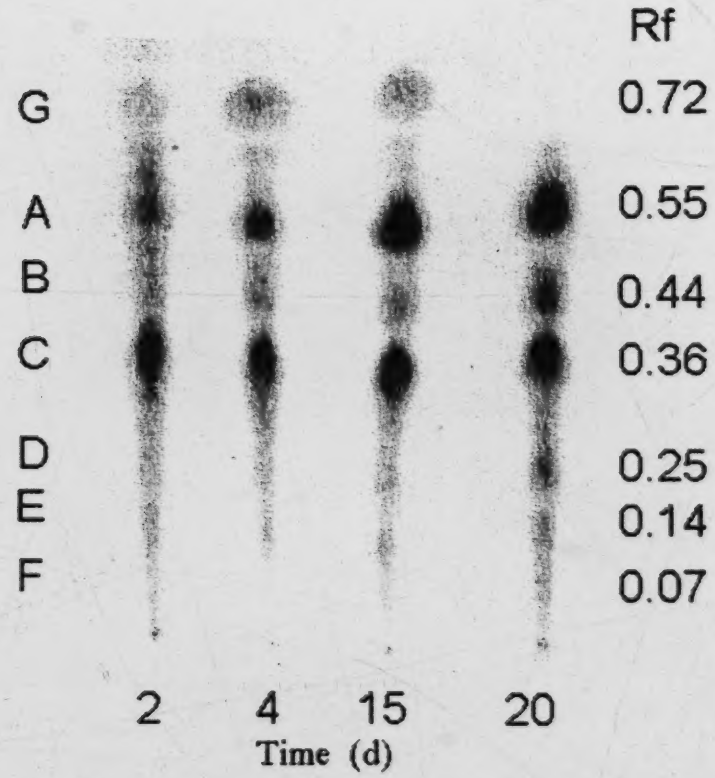

Figure 4.8 TLC of sophorolipids during batch fermentation of *C. bombicola* in the production medium.

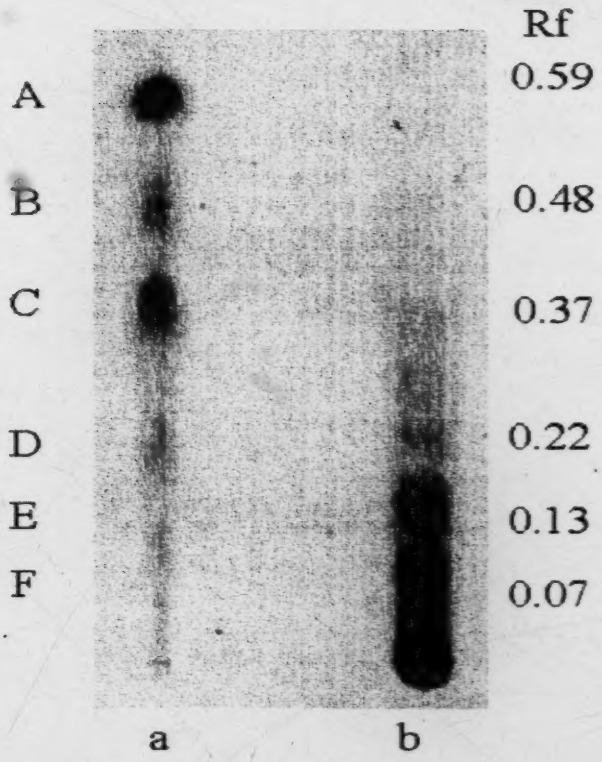

Figure 4.9 TLC of ethyl acetate extracted (a) and lyophilized (b) sophorolipids.

The composition of lactones within the ethyl acetate extracted sophorolipids was analyzed after 20 d of fermentation. LC separated the 20 d sophorolipids (6 g total) into five fractions and each fraction was analyzed gravimetrically and by TLC (Table 4.2 and Figure 4.10). The LC separated the lactone sophorolipids (A to D) well, but was ineffective in the separation the acidic sophorolipids. The first three fractions (0 % to 10 % methanol in chloroform) were sufficient to elute the majority of lactones. The total proportion of the lactones was calculated from the summation of the first three fractions and accounted for approximately 80 % of the ethyl acetate extracted sophorolipids. The

actual yield is likely higher, because some of the lactones remained within the last two fractions.

Table 4.2 Mass composition of sophorolipids in each LC fraction.

Fraction	Methanol/Chloroform (% v/v)	Mass (% m/m)
1	0	1
2	5	9
3	10	69
4	15	19
5	50	2

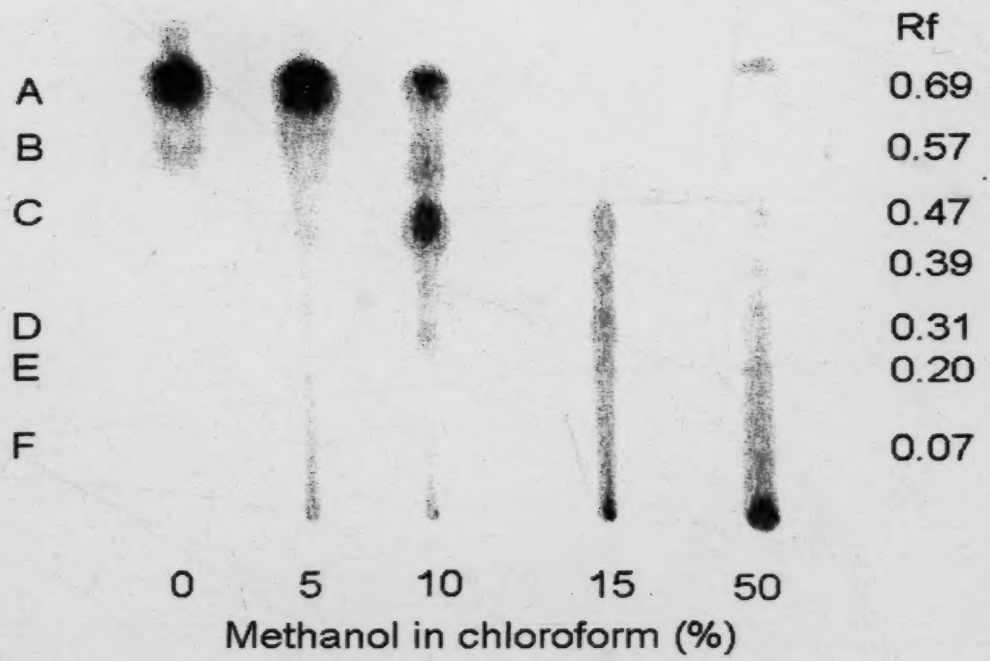

Figure 4.10 TLC of the 20 d sophorolipid mixture separated by the LC.

4.3.2 TLC and GC analysis of the contents of spot "G"

The 18:1 and 18:0 fatty acids were observed to be soluble and immiscible in ethyl acetate, respectively. The R_f values of 18:1 fatty acid and sophorolipids were compared. Spot "G" had an R_f value similar to 18:1 fatty acid providing a clue to its chemical nature (Figure 4.11). From this result, 18:1 fatty acid was used as the standard for the GC analysis of the spot "G".

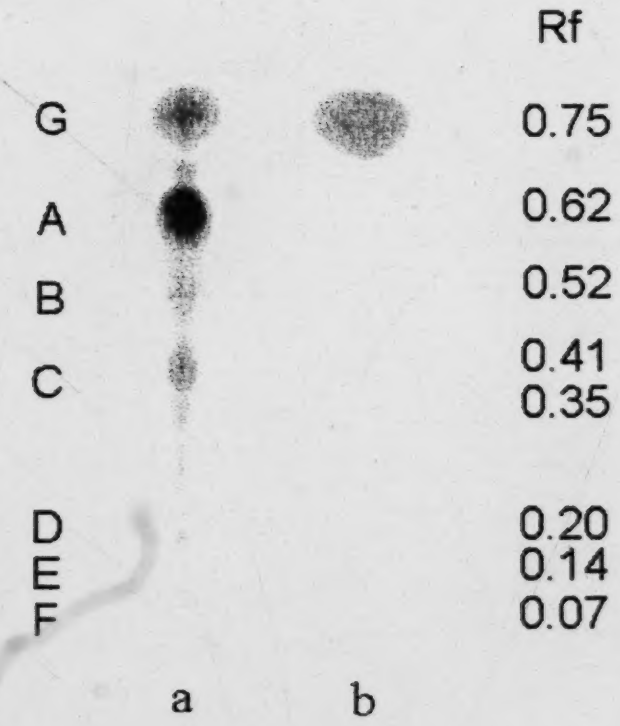

Figure 4.11 TLC of sophorolipids (a) and 18:1 fatty acid (b).

Sophorolipids from 15 d were loaded onto the preparative TLC and separated. Contents of spot "G" were scraped from the silica plate and eluted in ethyl acetate with flash LC. Diazomethane was used to convert the 18:1 fatty acid standard and spot "G" into FAME (Bressler and Fedorak, 2001). FAME was analyzed by GC-MS. The 18:1 FAME had a retention time of 31.18 min (Figure 4.12a). GC analysis of the contents of spot "G" showed at least nine different peaks (Figure 4.12b). Each peak was numbered in order of retention time. There were likely more peaks, because the GC did not separate peaks eight and nine very well. Peak five had a retention time of 31.11 min and was the closest to the 18:1 FAME. The area of each peak was used to compare the composition of the volatilized FAME. Peak five corresponded to 7 % of the sample and the peaks eight and nine constituted 78 % of the total volatilized FAME (Table 4.3). The GC analysis showed that spot "G" was found to contain other lipids and one of which (peak five) had a very similar retention time to the 18:1 FAME.

Table 4.3 Retention times and areas of the spot "G" FAME.

Peak	Retention time (min)	Area (%)
1	26.93	1.31
2	27.05	0.52
3	27.53	5.20
4	30.99	1.85
5	31.11	6.80
6	31.51	1.69
7	31.84	4.19
8	33.48	48.41
9	38.48	30.03

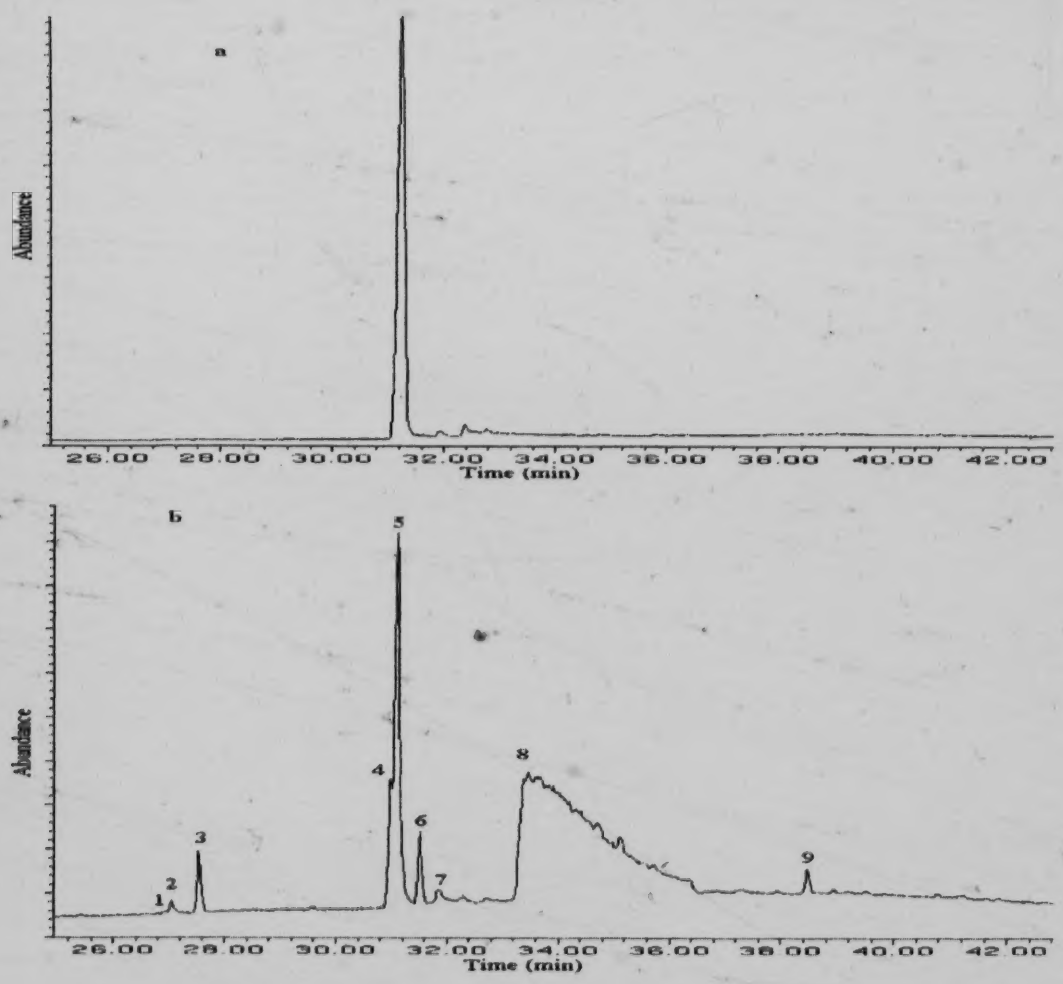

Figure 4.12 GC of methylated 18:1 (a) and spot "G" FAME (b).

4.3.3 GC-MS of components extracted from spot "G"

The mass spectrum of each peak in Figure 4.12 was determined and compared to the database. High database matches were observed for peaks four (99 %), five, (99 %) and six (90%). However, the structures of the remaining six peaks were undetermined, due to low matches with the database. Mass spectra of 18:1 FAME (Figure 4.13) and peak five (Figure 4.14) were determined. The base peak and parent ion for both were similar at m/z 55 and 296 respectively. Large fragments with m/z of 264, 222, and 180 were detected for both. This led to the conclusion that peak five was 18:1 FAME. Peak four matched closely to 18:2 FAME. The mass spectrum of peak four was determined and compared to 18:2 FAME (Figure 4.15 and Figure 4.16). The base peak and parent ion of both were similar of m/z 67 and 294 respectively. Large fragments of m/z 263, 234, 164, and 150 were detected. This led to the conclusion that peak four was likely 18:2 FAME.

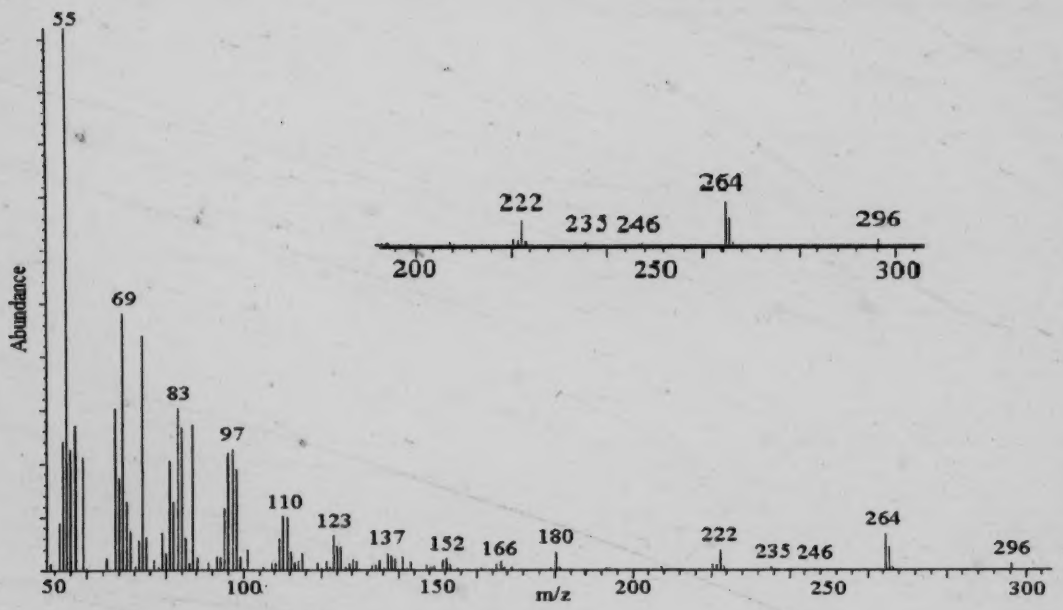

Figure 4.13 MS of 18:1 FAME (experimental).

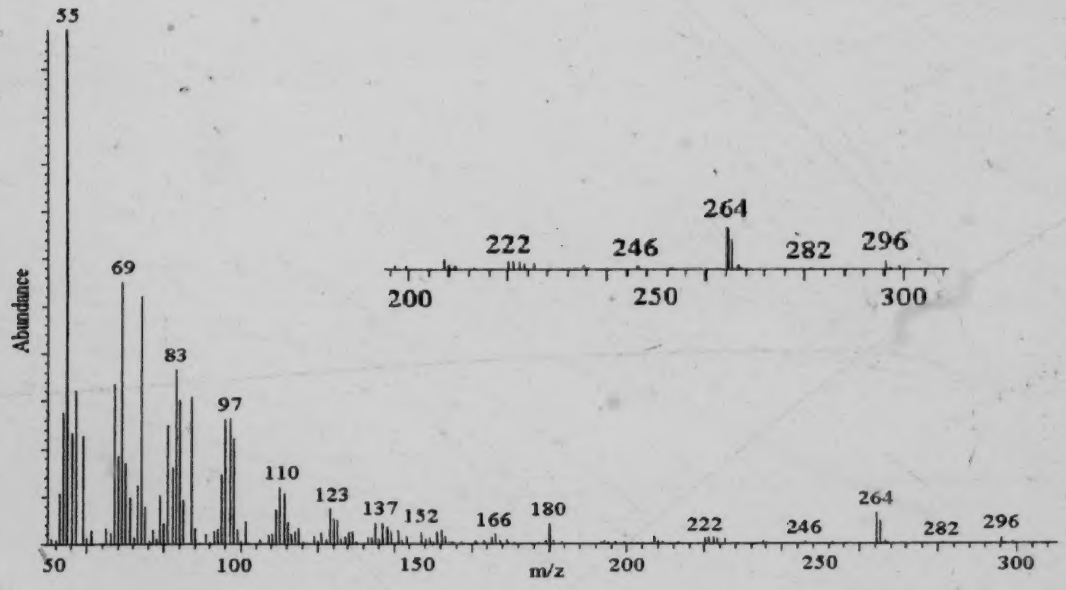

Figure 4.14 MS of peak five.

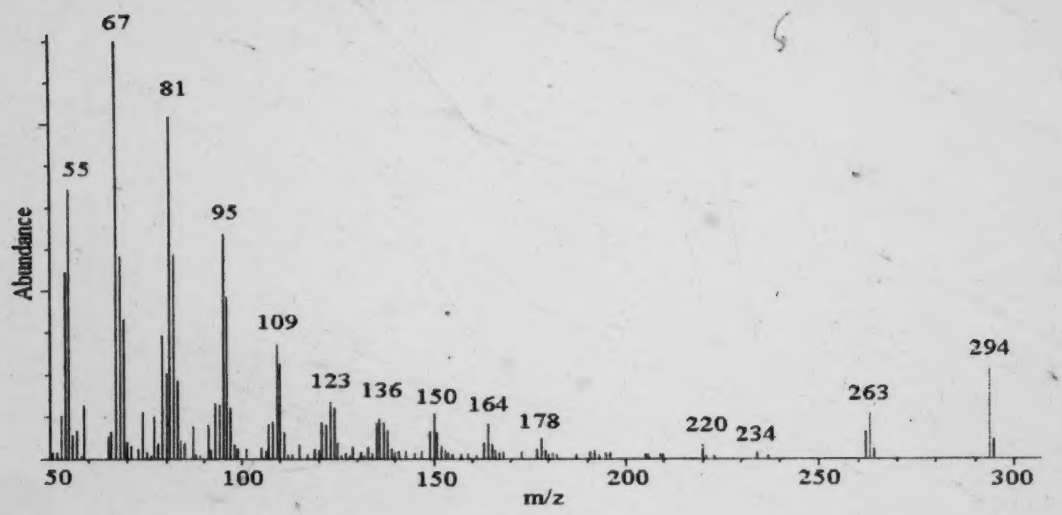

Figure 4.15 MS of 18:2 FAME (database).

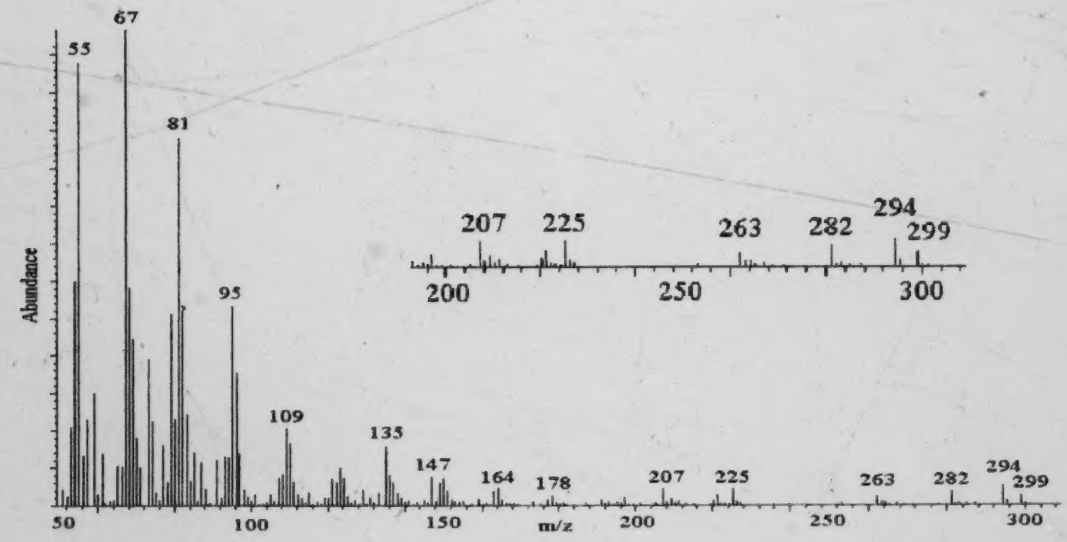

Figure 4.16 MS of peak four.

Peak six had a match of 90 % with 16-methylheptadecanoic acid methyl ester. However, the MS of peak six was compared to 18:0 FAME instead (Figure 4.18 and Figure 4.19), because the molecular weight of 18:0 FAME and 16-methyl heptadecanoic acid are similar at 298 g/mol. Moreover, branched chain fatty acids are not found in sunflower oil (Gunstone *et al.*, 1986). The base and parent ion peaks of 18:0 FAME and peak six were similar at m/z 74 and 298, respectively. Large fragments of m/z 255, 199 and 143 were detected. The intensities of fragments at m/z 55 (30 %), 87 (55 %), 143 (10 %), 199 (5 %) and 255 (5 %) matched were similar for 18:0 FAME and peak six. Thus the structure of peak six was tentatively identified as 18:0 FAME.

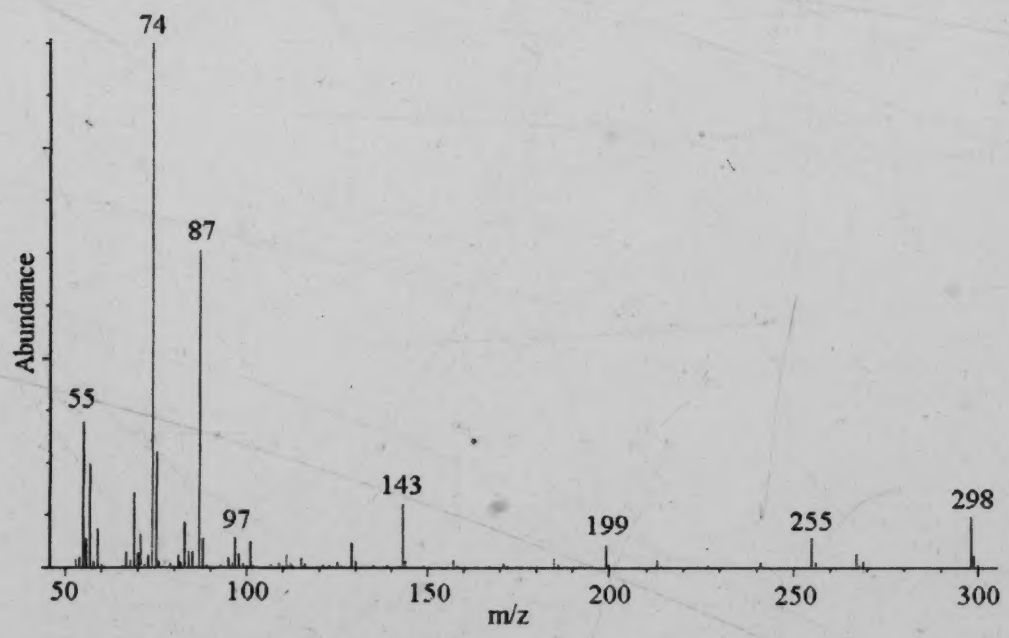

Figure 4.17 MS of 18:0 FAME (database).

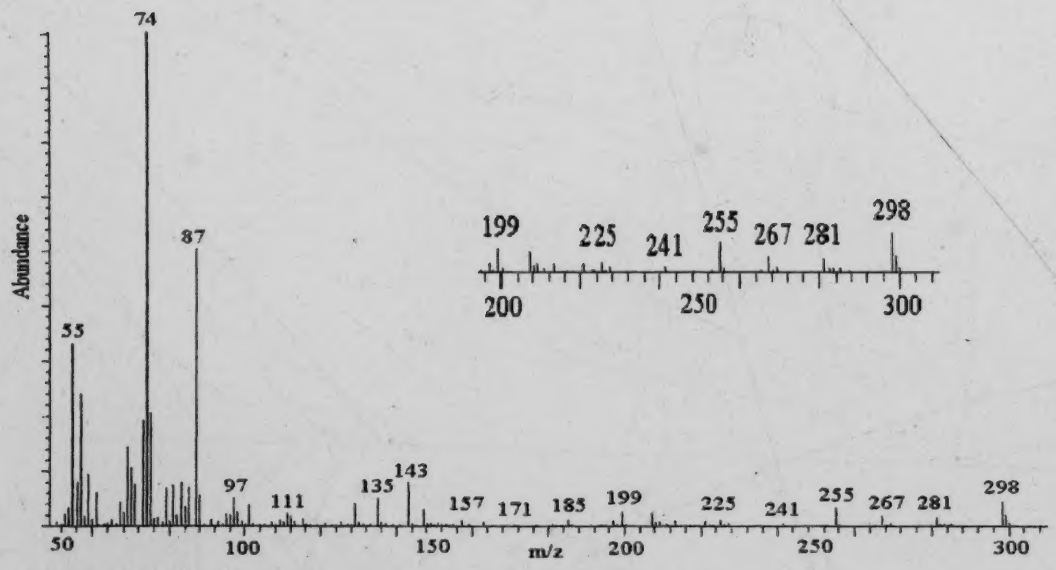

Figure 4.18 MS of peak six.

4.3.4 Interfacial properties of acidic sophorolipids

The 20 d sophorolipids containing both lactonic and acidic sophorolipids were converted to acidic sophorolipid by alkaline hydrolysis. Acidic sophorolipids were favored as starting material for semi-synthetic biosurfactants. The surface and interfacial properties of the acidic sophorolipid mixtures were determined in order to provide a starting point for these modifications.

CMC is one criterion used to evaluate the effectiveness of surfactants. CMC is the minimum concentration of surfactants required to form micelles, and corresponds to a sharp drop in the measured interfacial tension (Young and Coons, 1945). The CMC of the acidic sophorolipids was between 70 mg/L to 80 mg/L, and the minimum surface tension was 37 mN/m (Figure 4.19). In comparison acidic sophorolipids reduced the interfacial

tension between water and toluene from 36 mN/m to 2 mN/m (Figure 4.20). The CMC for toluene-water interface was between 90 mg/L to 100 mg/L. These results indicated that acidic sophorolipids were very effective interfacial tension reducers, while the opposite was true for surface tension. The overall solubility of acidic sophorolipids in water and toluene were very high in the order of 100 mg/L.

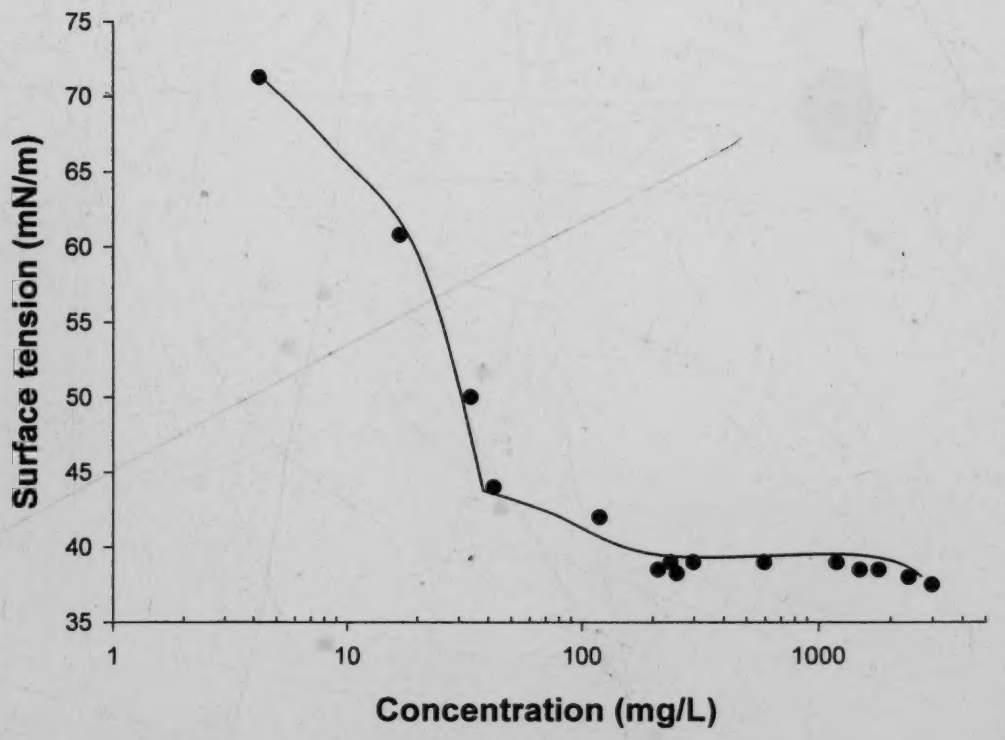

Figure 4.19 Surface property of acidic sophorolipids from *C. bombicola*. Lines are trends only.

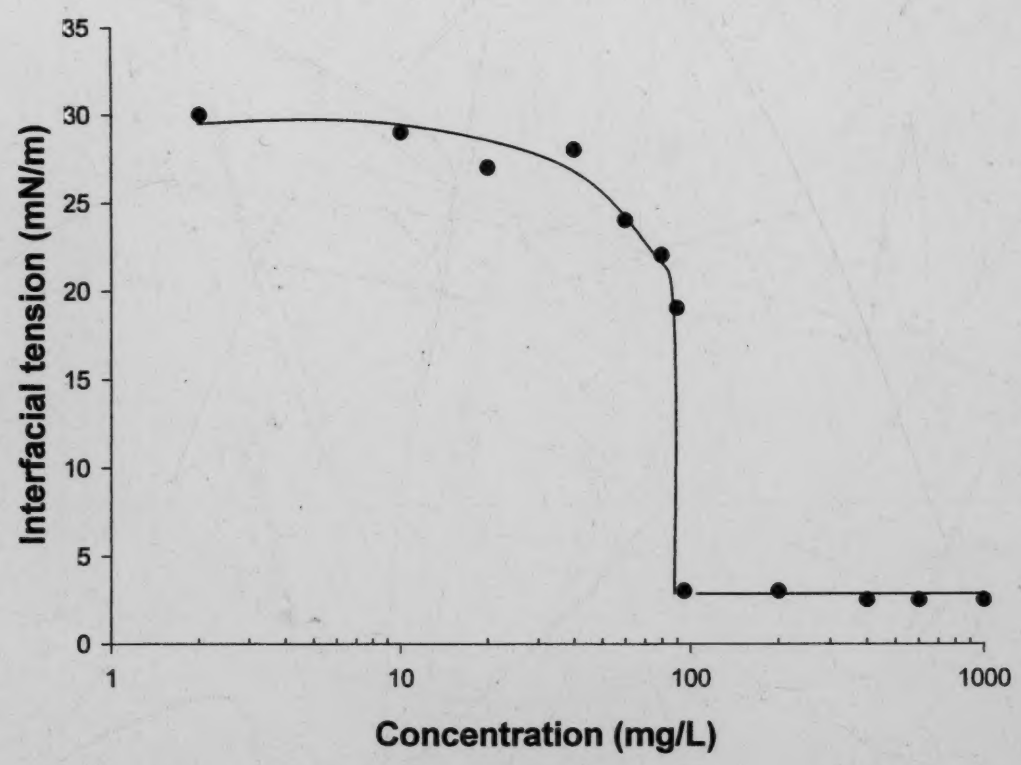

Figure 4.20 Interfacial property of acidic sophorolipids from *C. bombicola*. The interfacial tension was measured between water and toluene. Lines are trends only.

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5.0 Discussion

5.1 Fermentation of *C. bombicola*

The nature of sophorolipid production has been compared to secondary metabolites, because *C. bombicola* cultures are capable of producing these compounds during the stationary phase. Secondly, cells of *C. bombicola* in a nitrogen free medium have been shown capable of producing sophorolipids. These cultures are called resting cells (Gobbert *et al.*, 1984). In batch fermentations, sophorolipids are produced when the growth medium containing glucidic and lipidic carbon sources is depleted of nitrogen (Asmer *et al.*, 1988). Although not as common, sophorolipids have been produced under depletion of inorganic phosphate in the growth medium (Albrecht *et al.*, 1996).

An extended batch fermentation time of 20 d was used to analyze the later stages of cell growth, sophorolipid production and composition. The stationary phase of *C. bombicola* was reached after 2 d of growth in the inoculum medium. In comparison, the OD in the production medium continued to increase after 2 d of growth (Figure 4.5), likely due to the accumulation of sunflower oil and sophorolipids in the cell. Hommel *et al.* (1994) observed the accumulation of oil in the cytoplasm of similar yeast called *Candida apicola*. Zhou and Kosaric (1993) observed the presence of intracellular sophorolipids. OD decreased from 30 to 20 between 15 d and 20 d indicating that biomass has decreased after 15 d of fermentation. Decrease in OD has not been noted in batch fermentations of *C. bombicola*, because these are typically in the order of 3 d to 8 d (Klekner *et al.*, 1991, Lee and Kim, 1993, Garcia-Ochoa and Casas, 1999, Zhou *et al.*, 1992). Longer times of 12.5 d and 25 d have been noted for fed-batch fermentations (Daniel *et al.*, 1998a, Daniel

et al., 1998b). Biomass did not decrease below the stationary phase under these fermentation conditions, likely due to abundance of carbon source for cell energy maintenance. The decrease in pH from 6 to 3 observed in Figure 4.5 is typical of fermentations associated with *C. bombicola* (Asmer *et al.*, 1988, Deshpande and Daniels, 1995, Garcia-Ochoa and Casas, 1999, Ito and Inoue, 1980, Klekner *et al.*, 1991, Zhou *et al.*, 1992). The reduction of pH is partially due to secretion of citrate and isocitrate into the medium (Albrecht *et al.*, 1996). Acidic sophorolipids, acetic acid and other free fatty acids contribute to further pH reduction.

Garcia-Ochoa and Casas (1999) did not account for the consumption of sunflower oil in their unstructured kinetic model, because it was based on only three responses that included glucose, biomass and sophorolipids. Sunflower oil consumption (Figure 4.5) was determined in this study to show that the fate of vegetable oil in *C. bombicola* fermentation was an important factor in sophorolipid production. Any mathematical model of sophorolipid production would have to account for the fate of the lipidic substrate used, because the lipidic substrate directly determines the fatty acid backbone of sophorolipids (Davila *et al.*, 1994, Gorin *et al.*, 1961, 1962; Tulloch *et al.*, 1962, 1968; Tulloch and Spencer, 1967, 1972; Heinz *et al.*, 1969, 1970, Spencer *et al.*, 1979). The maximum rates of sophorolipid production and oil consumption were between 0 d to 8 d. The depletion of sunflower oil after 8 d likely led to a decrease in the rate of sophorolipid production.

The consumption profiles of several fatty acids were analyzed in this work (Figure 4.6 and Figure 4.7). The motivations for these analyses were to provide new data for future models and to determine if any of the fatty acids were favored for consumption within the sunflower oil. The 18:1 fatty acid is favored for the production of sophorolipids (Asmer *et al.*, 1988). More specifically, production of diacetylated lactones tended to be favored by vegetable oils with high content of 18:0 and 18:1 fatty acids (Davila *et al.*, 1994). Results in this work showed that initial preference of *C. bombicola* for fatty acids within the sunflower oil were largely determined by their abundance in the production medium (Figure 4.6). High initial rates of consumption tended to favor the least abundant fatty acids. However, the normalized concentrations of fatty acids after 15 d were all similar indicating that despite high initial rates, the overall consumption rate of fatty acids was the same. This outcome was unexpected, because all fatty acids were expected to have similar rates of consumption, if bulk consumption of the sunflower oil was occurring. Further experimentation would have to be done to test the validity of this result.

Concentrations of some fatty acids appeared to fluctuate greatly during the production of sophorolipids (Figure 4.7). In this figure the concentrations of the fatty acids were normalized to C_{max} instead of C_0, because the concentration of some fatty acids (24:0 and 22:0) were higher after 0 d. The initial concentration of 24:0 fatty acid was below the detection limit at 0 d, but increased from 0 d to 15 d of sophorolipid production. The level of 22:0 fatty acid increased from 0 d to 6 d and decreased to the initial level after 15 d. The increases in both fatty acids could not be attributed to error, because their concentrations after 0 d were above the detection limit. Thus increases in 22:0 and 24:0

and fluctuations in 20:0 and 22:1 observed in Figure 4.7 is likely due to cellular production. More specifically, the extraction of sunflower oil was also extracting some fatty acids associated with *C. bombicola*.

The possible sources of the fluctuations in 20:0, 22:0, 22:1, and 24:0 fatty acids were not determined, but three possible sources of these fatty acids are cytoplasm, lipid membrane and the extracellular environment. Hexane extraction did not show a significant reduction in biomass (Table 4.1), when *C. bombicola* was grown in the absence of sunflower oil, indicating that the methodology used did not produce leaky cells. Other authors have also shown that hexane tended to partition in the center of lipid bilayers (White *et al.*, 1981). Therefore it is unlikely that the fatty acids obtained from hexane extraction were from the cytoplasm of *C. bombicola*. The fatty acids are likely associated with the cell membrane or found in the extracellular medium.

A probable explanation for the increase in 22:0 and 24:0 fatty acids is that *C. bombicola* produces these fatty acids in response to the stress presented by the sophorolipids and other organic acids present in the production medium. Natural lipophilic solvents such as vegetable oils are not toxic to whole cells (Heipieper *et al.*, 1994). However, acids and alcohols can increase the fluidity of the cell membrane. Examples of organic compounds that can be found in the *C. bombicola* production medium are sophorolipids, fatty acids, citric acid, acetic acid, and glycerol (Albrecht *et al.*, 1996). Adaptation of microorganisms such as *Escherichia coli* and *Pseudomonas putida* to hydrocarbons have been to increase the rigidity of the cell membrane by such methods as increasing cis-

mono unsaturated fatty acids and length of fatty acid chains (Heipieper *et al.*, 1994). This explanation would also be consistent with observations of baker's yeast grown in medium containing isooctane (Kawamoto *et al.*, 2001). The authors discovered that the composition of the lipid membrane of *Saccharomyces cerevisiae* changed in response to the stress presented by isooctane. More specifically the rigidity of the cell membrane increased in response to the presence of isooctane. Lastly, these authors showed that changes in the fatty acid composition of the yeast lipid membrane led to an increase in tolerance of these yeasts in environments containing hydrocarbons. Thus *C. bombicola* cells could be adapting to the stress presented by several organic compounds mentioned above by increasing the rigidity of the cell membrane. Increasing the content of long chain fatty acids such as 22:0 and 24:0 during the growth is one mechanism to increase the rigidity of the cell membrane.

5.2 Biomass determination and product yield coefficients

Ethyl acetate had a significant effect on the measurement of DCM of *C. bombicola.* More specifically, ethyl acetate was shown to cause leakiness of *C. bombicola* cell membranes leading to a reduction in biomass (Table 4.1 and Figure 4.1). Sophorolipids are found intracellularly (Zhou and Kosaric, 1993), making the DCM measurement of biomass inaccurate if done prior to sophorolipid extraction. The measured biomass in this study was reduced by 34 % after the liquid broth was extracted with ethyl acetate. The effect of solvent extraction on the biomass was comparable to the results of Lee and Kim (1993). These authors found a reduction of 22.4 % in biomass after extraction with ethyl acetate. OD was used to measure biomass during the fermentation of *C. bombicola* in the

inoculum and production medium, because DCM would not have been more accurate than OD. It was also possible to estimate a crude biomass measurement of *C. bombicola* after 20 d of cultivation by using the correlation that OD 20 is equivalent to 6 g/L of biomass. This correlation was directly deduced from Figure 4.1 and Figure 4.4. A future consideration for estimating the biomass of *C. bombicola* would be to use its protein content, because sophorolipids do not have nitrogen.

One focus of this study was to understand the nature of sophorolipid production. Several authors have studied various production techniques in increasing sophorolipid production (Table 1.2). The growth and production conditions used in this study were based on the work of Garcia-Ochoa and Casas (1999). These conditions were chosen for the simplicity of the production medium that contained glucose, sunflower oil, yeast extract, and water. More importantly, high sophorolipid production was possible, as long as the production medium contained both glucidic and lipid carbon sources. There were very few differences from the experimental set up and those of the previous authors. The differences in this study were such that YM was used as the inoculum medium, cultures were cultivated in shake flasks and OD was used to measure biomass during the fermentation of *C. bombicola.*

The production of sophorolipids in batch fermentations has a typical range of 6.5 g/L to 137 g/L (Table 1.2). The sophorolipid and biomass yields in this study were 45 g/L and 6 g/L, respectively. The majority of authors have increased the overall yield of sophorolipids by manipulating the formulation of the growth and production medium.

Other production conditions such as pH control, aeration and agitation have also been studied. Nonetheless a wide range of production levels exists, despite the optimization of sophorolipid production.

Yield coefficients can be used to determine the fate of particular carbon based substrates in fermentations. In fermentations with secondary products (sophorolipids), the simplest relationship indicates that all substrates consumed end up in biomass, product and carbon dioxide shown by Equation 5.1 (Bailey and Ollis, 1985; Lee and Kim, 1993; Garcia-Ochoa and Casas, 1999). The limitation of this equation is other possible products are lumped into biomass, sophorolipids or carbon dioxide. For example, citrate and acetate are excreted into the production medium by *C. bombicola* (Albrecht *et al.*, 1996), but are not any of the three variables mentioned in above. As shown in this study, a large mass equivalent to 25 % of extracted sophorolipids was not extracted by ethyl acetate. This unaccounted mass would be lumped into the variable not analyzed to satisfy the equation, in this case the yield coefficient of carbon dioxide. Despite these limitations, Equation 5.1 can be used to compare the $Y_{P/S}$ and $Y_{X/S}$. However, caution is necessary when this equation is used to obtain $Y_{CO2/S}$. An interesting trend occurred when $Y_{P/S}$ and $Y_{X/S}$ of different authors were plotted. Yield coefficients of *C. bombicola* in batch fermentations were obtained from several studies (Ito *et al.*, 1980 Ito and Inoue, 1982; Cooper and Paddock, 1984; Zhou *et al.*, 1992, Zhou and Kosaric, 1993; Lee and Kim, 1993; Deshpande and Daniels, 1995; Garcia-Ochoa and Casas, 1999).

Equation 5.1 Mass balance of yield coefficients

$$Y_{\frac{p}{s}} + Y_{\frac{x}{s}} + Y_{\frac{CO_2}{s}} = 1$$

$$Y_{\frac{p}{s}} = \frac{mass_sophorolipids_produced}{mass_substrate_consumed}$$

$$Y_{\frac{x}{s}} = \frac{mass_biomass_produced}{mass_substrate_consumed}$$

$$Y_{\frac{CO_2}{s}} = \frac{mass_carbon_dioxide_produced}{mass_substrate_consumed}$$

The comparison of the $Y_{P/S}$ and $Y_{X/S}$ produces a simple correlation that describes the overall result of each study involving batch fermentation of sophorolipids (Figure 5.1). In this study the $Y_{P/S}$ and $Y_{X/S}$ were 0.25 and 0.033 respectively. Other restrictions applied to this correlation are such that both lipidic and glucidic carbon sources are necessary and an organic nitrogen source is included in the medium. The lipidic source is from nature such as vegetable oils or animal fats. Glucose is used for the glucidic carbon source. The inoculum for the production medium must be a fixed volume from a liquid growth medium and not a loop of cells from an agar plate as used by Klekner *et al.* (1991). The use of a single loop of cells led to a much lower $Y_{P/X}$ value of 0.57 (Klekner *et al.*, 1991). This low $Y_{P/X}$ indicates that a higher biomass of *C. bombicola* was produced than sophorolipids. Other parameters such as pH, aeration, agitation, temperature, inorganic nitrogen, other trace minerals used by different authors do not need to be similar for this correlation to be valid.

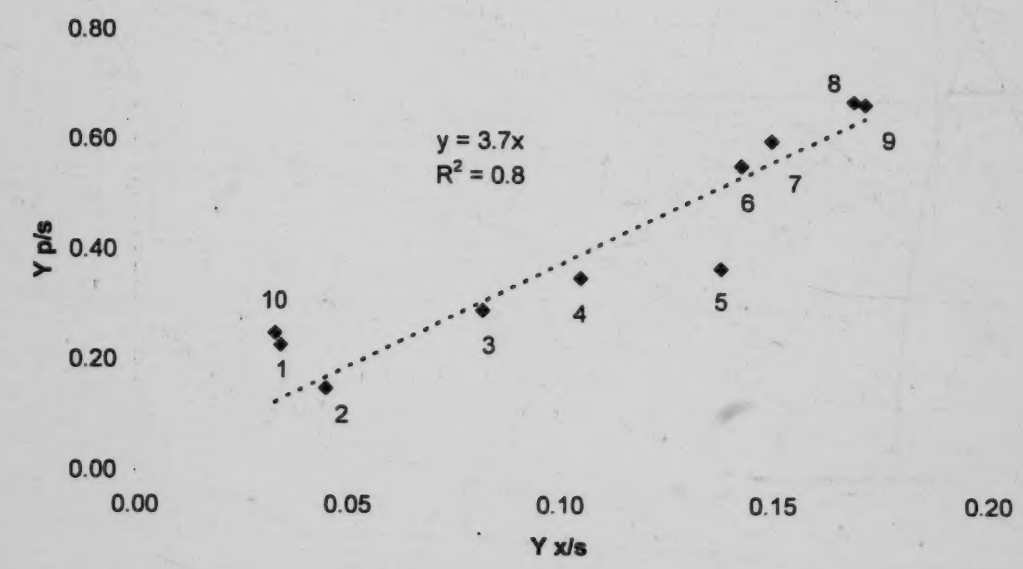

Figure 5.1 $Y_{P/S}$ and $Y_{X/S}$ for batch fermentations of *C. bombicola*. No te: (1) Ga rcia-Ochoa and Casas (1999), (2) Ito *et al.* (1980), (3) Ito and Inoue (1982), (4) Cooper and Paddock (1984), (5) Lee and Kim (1993), (6) and (7) Deshpande and Daniels (1995) without and with pH control, (8) Zhou and Kosaric (1993), (9) Zhou *et al.* (1992), (10) this study.

The regression in Figure 5.1 shows that $Y_{P/X}$ is 3.7. More importantly this indicates that the increase in sophorolipids achieved by each author is the direct result of an increase in the biomass produced by *C. bombicola* in the production medium. This idea is not novel for batch fermentations of a microorganism in the same medium and batch fermentation conditions (Pirt, 1975). More specifically, several authors have increased the sophorolipid yield within the constraints of their medium formulations by simply increasing the glucose concentration and oil in the production medium (Casas and Garcia-Ochoa, 1999 Cooper and Paddock, 1984, Klekner *et al.*, 1991, Spencer *et al.*, 1979, Zhou and Kosaric, 1995, Zhou *et al.*, 1992). Increasing the concentration of the carbon sources has been one aspect of the optimization of the production medium of *C. bombicola*. Nonetheless, a comparison between optimized production media led to various sophorolipid yields from as low as 4 % (m/v) to as high as 13.7 % (m/v) (Table 1.3). The application of Equation 5.1 to generate Figure 5.1 is unique in this work, because all studies analyzed have essentially their own unique medium formulation, growth and production conditions. This correlation shows that optimization of the production medium is not as essential as increasing the biomass prior to sophorolipid production.

5.3 Extraction of sophorolipids and sunflower oil

The liquid broth of *C. bombicola* during the over production of sophorolipids was composed of three phases (from top to bottom): oil, water and sophorolipid. Two different solvents were necessary to extract the oil and sophorolipids from the mixture. Tulloch and Spencer (1972) were the first to use ethyl acetate to extract sophorolipids

from *C. bombicola*. Ito *et al.* (1980) improved this technique and used hexane to extract any unconverted oil prior to extraction with ethyl acetate. Oil is very soluble in hexane, while the sophorolipids were very soluble in ethyl acetate. Each compound was poorly soluble in the other solvent. Several authors used and adapted the method of Ito *et al.* (1980) (Cooper and Paddock, 1984; Inoue and Ito, 1982; Ito and Inoue, 1982; Lee and Kim, 1993; Deshpande and Daniels, 1995; Casas *et al.*, 1997; Casas and Garcia-Ochoa, 1999). Other authors used different solvents to extract oil and sophorolipids from the biomass. Asmer *et al.* (1988) used a mixture of ethanol, butanol and chloroform (10/10/1, v/v/v) to remove the oil and sophorolipids from the aqueous phase. Zhou *et al.* (1992) used methanol and chloroform (10/1, v/v), while Rau *et al.* (1996) used ethanol and butanol (1/1, v/v). Most authors have not accounted for the effects of solvent extraction on the measurements of oil, biomass and sophorolipids. Lee and Kim (1993) were the exception, because the authors accounted for the effect of ethyl acetate on the biomass. The solvent extraction led to a 22.4 % loss in the measured biomass (Lee and Kim, 1993).

After four extractions with ethyl acetate, a sophorolipid phase was still present in the liquid broth. TLC analysis of this phase showed that some spots had R_f values similar to acidic sophorolipids (Figure 4.9). Acidic sophorolipids were found to be poorly soluble in ethyl acetate. However, a large proportion of this phase was not separable by the TLC method used. Gravimetric analysis indicated that the content of this phase was equivalent to 25 % of the ethyl acetate extracted sophorolipids. The results in this study indicated that ethyl acetate was biased towards lactone sophorolipids. Some acidic sophorolipids

are likely not extracted by ethyl acetate. Ideally, one solvent would suffice to extract both lactone and acidic sophorolipids. Lactone sophorolipids are more hydrophobic than acidic sophorolipids (Gorin *et al.*, 1961, 1962). This difference in chemical properties of lactone and acidic sophorolipids make it improbable to use one solvent for the extraction of all types of sophorolipids. In this study, the remaining sophorolipid phase that was insoluble in ethyl acetate was simply lyophilized. This method would be inadequate to select against water-soluble organics such as glucose and sophorose that are also soluble with acidic sophorolipids. The use of two solvents for the extraction of sophorolipids in succession is an option. In conjunction with ethyl acetate, pentanol can be used afterwards to extract the remaining acidic sophorolipids. Pentanol had been previously shown to favor the extraction of acidic sophorolipids over lactone sophorolipids (Deshpande and Daniels, 1995).

The use of hexane to extract the sunflower oil was found to be sufficient. The majority of the sunflower oil contained mainly 18:3, 18:1, 16:0, and 18:0 fatty acids. The measured composition of the major fatty acids in the sunflower oil was minimally affected by hexane extraction. The total minor fatty acids was less than 5 % of the sunflower oil. The effect of hexane on the composition of the minor fatty acids was studied, because future studies on the consumption of oil would have to account for the fate of all fatty acids. The minor fatty acids affected were 16:1, 20:1, 20:2, 22:0, and 22:1 fatty acids. The hexane extract showed higher values for 16:1 and 20:1 fatty acids than expected. In comparison lower values were observed than expected for 20:2, 22:0 and 22:1. These results indicated that hexane had a slight bias with the five minor fatty acids.

5.4 Purity of sophorolipids and FAME analysis

Asmer *et al.* (1988) first published the TLC of sophorolipids. The mixture of sophorolipids observed in this study was identified according to Asmer *et al.* (1988). As discussed above, the use of ethyl acetate for sophorolipid extraction was biased towards extracting lactonic sophorolipids (Figure 4.9). Thus it was not surprising that the majority of sophorolipids analyzed during *C. bombicola* fermentation were lactonic sophorolipids (Figure 4.8). A LC method was used to separate sophorolipids from a 20 d culture and TLC was used to determine the proportion of lactonic sophorolipids. Gravimetric analysis in conjunction with TLC of the separated sophorolipids indicated that lactonic sophorolipids constituted approximately 80 % of ethyl acetate extractable sophorolipids (Figure 4.10). This estimation was achieved by the summation of the percent mass of the first three LC fractions (Table 4.2), because these fractions gave the darkest TLC spots for lactonic sophorolipids (Figure 4.10). This result also showed that acidic sophorolipids did not separate very well under the conditions used, because TLC of the last two fractions were plagued with smears and dark spots at the point of origin.

One missing element in the study of sophorolipid production was an analysis on the purity of these sophorolipids. Davila *et al.* (1993) have shown that sophorolipids comprised of a mixture of nine related compounds. Asmer *et al.* (1988) also have shown that several "unknown lipids" were found with the sophorolipid mixture when *C. bombicola* was cultivated in 18:1 fatty acid containing medium. However, the composition of these "unknown lipids" has not been analyzed. In this study, one TLC

spot l abeled s pot " G" h ad a similar R_f value to one of these "unknown lipids". The presence of the spot "G" during the fermentation of *C. bombicola* was thus compared to the results of Asmer *et al.* (1988). These authors noticed two to three spots with R_f values greater than the diacetyl lactone sophorolipid (spot A) when *C. bombicola* was cultivated in 18:1 fatty acid with and without glucose. Klekner *et al.* (1991) also showed their TLC results, but the sophorolipids were not eluted to an adequate separation and the TLC plate was overloaded. Unfortunately most studies have not reported the composition of sophorolipids and have instead focused on the absolute concentration of sophorolipids. The a uthors that have used TLC to study the composition of sophorolipids have not shown the actual TLC and instead have indicated the R_f values concerning sophorolipids (Cooper and Paddock, 1984; Brakemeier *et al.*, 1997; Daniel *et al.*, 1998a, 1998b; Otto *et al.*, 1999). The complete consumption of sunflower oil after 20 d of fermentation likely led to the disappearance of the spot "G" (Figure 4.5 and Figure 4.8). It should be noted that a very minute amount of spot "G" likely existed, and its disappearance was related to the detection limit of the experiments. The overlying significance of the presence of other lipid products within the sophorolipids is that the quality of the sophorolipids was compromised. More specifically, very long fermentation times in the order of 20 d were necessary to obtain sophorolipids with high purity. This result has its main repercussion in the prohibitive cost of long fermentation times. However, the alternate of perpetuating the spot "G" in shorter fermentation times would incur further costs in any downstream processing that would remove the content of spot "G". A balance would be necessary in the guarantee of a pure product of sophorolipids and the cost of attaining that level of

purity. Further studies would be necessary to establish a cost analysis of the removal of this component from sophorolipids.

GC analysis of spot "G" FAME showed that this spot was found to consist of at least nine compounds (Figure 4.12b). MS analysis identified 18:1 fatty acid and tentatively identified 18:2 and 18:0 fatty acids within the contents spot "G". Unfortunately, the remaining six compounds were not identified by GC-MS. One peak with a retention time of 33.48 min interacted non-ideally with the GC column leading to a large "shark fin" peak (Figure 4.12b). This peak could be free fatty acids that have not been converted to FAME (Fedorak, personal communication). The non-ideal nature of this "shark fin" peak led to a very weak match with the MS database. Further refinement of column and GC conditions would likely elucidate the chemical nature of this and other peaks. The GC area for peak nine was strangely large (30 % of the total) in comparison to the size of this peak (Table 4.3 and Figure 4.12b). This large area is likely a result of an integration error, and should not be considered as accurate. The database match between peak six and 16-methylheptadecanoic FAME was likely due to the limitation of some FAME within the database. As indicated in the results, sunflower oil does not have branched fatty acids (Gunstone *et al.*, 1986).

The identification of 18:1, and tentative identification of 18:0 and 18:2 fatty acids were not surprising at first, because these fatty acids could be a small amount of sunflower oil co-extracted with the sophorolipids. However, food grade vegetable oils do not generally have free fatty acids (Gunstone *et al.*, 1986). Moreover, the mass obtained from ethyl

acetate extraction of the production medium at 0 d was well below the detection limit of 0.1 g/L. Subjecting this extract to TLC did not produce any spots (data not shown), indicating that any free fatty acids in this extract was not detectable. These free fatty acids could have still come from the sunflower oil, but only after cleavage from the oil by a lipase enzyme. Lipases are capable of cleaving ester bonds of triacyl glycerols, thus freeing the fatty acids (Gunstone *et al.*, 1986). More specifically, other authors have postulated that *C. bombicola* cells produce lipases, because these enzymes have been observed in a similar organism called *Candida* apicola (Rau *et al.*, 2001; Hommel *et al.*, 1994d). The presence of lipase from *C. bombicola* would not be surprising, because this organism has been shown to metabolize a variety of vegetable oils from canola, safflower, soybean, and sunflower (Zhou and Kosaric, 1993; Ito and Inoue, 1980, Ito *et al.*, 1982; Zhou *et al.*, 1992; Gobbert *et al.*, 1984; Lee and Kim, 1993; Cooper and Paddock, 1984; Klekner *et al.*, 1991; Casas and Garcia-Ochoa, 1999; Garcia-Ochoa and Casas, 1999). Thus it is probable that 18:0, 18:1 and 18:2 fatty acids are products from the cleavage of the sunflower oil by lipases. Further experiments would have to confirm the presence of a lipase enzyme from *C. bombicola*.

5.5 Surface and interfacial tensions

Acidic sophorolipids are favored as starting material for the synthesis of semi-synthetic biosurfactants. In this study the effectiveness of acidic sophorolipids in reducing surface and interfacial tensions was analyzed. Several authors have studied the effects of sophorolipids on surface and interfacial tension (Cooper and Paddock, 1984; Brakemeier *et al.*, 1995, 1998; Otto *et al.*, 1999; Rau *et al.*, 1999). The CMC is the solubility of a

surfactant within an aqueous phase or the minimum surfactant concentration required for reaching the lowest interfacial and surface tension values (Lin, 1996). The CMC of the acidic sophorolipids was between 70 mg/L to 80 mg/L, while the minimum surface tension was 37 mN/m (Figure 4.19). This result was consistent with the literature values (Cooper and Paddock, 1984; Brakemeier *et al.*, 1995, 1998; Otto *et al.*, 1999; Rau *et al.*, 1999). More specifically, Rau *et al.* (1999) observed that the CMC and minimum surface tension of acidic sophorolipids were 75 mg/L to 85 mg/L and 40 mN/m, respectively.

The acidic sophorolipids were capable of reducing the interfacial tension of toluene-water from 36 mN/m to 2 mN/m. The CMC between toluene-water was approximately 90 mg/L to 100 mg/L (Figure 4.20). Hexadecane is generally used when measuring the interfacial properties of sophorolipids (Cooper and Paddock, 1984; Rau *et al.*, 1999). In this study toluene was used instead, to determine if the results were comparable to hexadecane. A mixture of acidic and lactonic sophorolipids can reduce the interfacial tension of water-hexadecane to 2 mN/m (Cooper and Paddock, 1984). More specifically, acidic sophorolipids have been shown to reduce the interfacial tension of water-hexadecane from 45 t o 8 m N/m (Rau *et al.*, 1999). The results of this study showed that acidic sophorolipids are also effective at reducing interfacial tension of toluene-water. Toluene is one of the most common aromatic pollutants found in the environment (Maier *et al.*, 2000). Further studies would be required to determine if the interfacial tension of other common aromatic pollutants such as benzene, ethyl benzene and xylenes could be reduced by sophorolipids.

6.0 Summary and conclusions

Maximum production of sophorolipids was successfully achieved after 20 d of batch fermentation. The final concentration of sophorolipids and biomass were 45 g/L and 6 g/L. The calculated $Y_{P/S}$ and $Y_{X/S}$ were 0.25 and 0.033, respectively and compared to values obtained by other authors (Figure 5.1). This figure showed a constant $Y_{P/X}$ of 3.7. This outcome indicates that increasing the density of *C. bombicola* culture in the production medium is more important for high a yield of sophorolipids, than optimizing media formulations or fermentation conditions. Different techniques of sophorolipid production would have to be used in order to obtain the $Y_{P/X}$ above 3.7. Fed-batch and resting cell methods are two possible options to batch fermentations that could possibly increase $Y_{P/X}$ of 3.7.

Lactones comprised approximately 80 % (m/m) of the ethyl acetate extracted sophorolipids from the 20 d culture (Figure 4.10). Caution is necessary when interpreting the composition of sophorolipids, because ethyl acetate tended to favor the extraction of lactone sophorolipids. Acidic sophorolipids were poorly soluble with this solvent. The solvent 1-pentanol can be used after ethyl acetate to extract any remaining acidic sophorolipids. A small sophorolipid phase was still observed after four extractions with ethyl acetate. This mass of this residue was equivalent to 25 % (m/m) of the ethyl acetate extracted sophorolipids. Unfortunately, the TLC method used did not separate all constituents of this residue. Further analysis would be necessary to conclusively identify the constituents of this residue.

Fermentation of 20 d was necessary to ensure high purity of sophorolipids, because sophorolipids obtained before 20 d contained other lipid products within the TLC spot "G" (Figure 4.8). This spot had a similar R_f position as the "unknown lipids" identified by other authors (Asmer *et al.*, 1988). GC analysis of the contents of spot "G" at 15 d of fermentation led to the detection of a mixture of at least nine compounds (Figure 4.12). MS analysis of the contents of spot "G" identified 18:1 fatty acid and tentatively identified 18:2 and 18:0 fatty acids (Figure 4.14, Figure 4.16 and Figure 4.18). The 18:1 fatty acid was identified after 15 d, indicating that longer fermentation times were necessary for complete consumption of this fatty acid, despite being favored for sophorolipid production (Tulloch *et al.*, 1970; Asmer *et al.*, 1988). The presence of 18:0, 18:1 and 18:2 free fatty acids in the production could be indicative of lipase activity. The identification of other peaks within spot "G" would be significant in further understanding the nature of sophorolipid production and oil consumption of *C. bombicola*.

The consumption of various fatty acids within the sunflower oil showed that initially, rates of consumption were highest for the least abundant fatty acids (Figure 4.6). However, all fatty acids were equally consumed after 15 d of production. This indicated that there was no overall preference for some fatty acids, despite 18:1 fatty acid being favored for sophorolipid production (Tulloch *et al.*, 1970, Asmer *et al.*, 1988). The consumption profiles of some fatty acids fluctuated greatly during sophorolipid production. In particular, 24:0 fatty acid increased (Figure 4.7) during production of sophorolipids, suggesting that this fatty acid is produced by *C. bombicola*.

Lastly, acidic sophorolipids were capable of reducing toluene-water interfacial tension from 36 mN/m to 2 mN/m with a CMC of 90 mg/L to 100 mg/L. Hexadecane is generally used when measuring the interfacial properties of sophorolipids (Cooper and Paddock, 1984; Rau *et al.*, 1999). This reduction in interfacial tension of toluene-water was comparable to the effect of acidic sophorolipids in hexadecane-water interfaces. The benefit of reducing the toluene-water interfacial tension is that this presents possible applications to biosurfactants used in environments contaminated with aromatics such as benzene, toluene, ethyl benzene and xylenes.

7.0 References

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